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Kupe Virus, a New Virus in the Family *Bunyaviridae*, Genus *Nairovirus*, Kenya

Mary B. Crabtree, Rosemary Sang, and Barry R. Miller

We have previously described isolation and preliminary identification of a virus related to Dugbe virus (DUGV), family *Bunyaviridae*, genus *Nairovirus*. Six isolates of the virus were obtained from pools of *Amblyomma gemma* and *Rhipicephalus pulchellus* ticks collected from hides of cattle in Nairobi, Kenya, in October 1999. We report results of further characterization of this virus, including growth kinetics in cell culture and full-length genome sequencing and genetic characterization, which show it to be distinct from DUGV. We suggest that this is a new virus in the family *Bunyaviridae*, genus *Nairovirus*, and we propose that it be designated Kupe virus.

The genus *Nairovirus* in the family *Bunyaviridae* comprises 7 species groups containing primarily tick-borne viruses, some of which have been identified as human or animal pathogens. The genome of the nairoviruses consists of 3 segments of negative-sense, single-stranded RNA, small (S), medium (M), and large (L), which encode the nucleocapsid protein, glycoproteins (Gn and Gc), and viral polymerase, respectively. Additionally, an M segment–encoded nonstructural protein, NS_M, was recently identified in the nairovirus Crimean-Congo hemorrhagic fever virus (CCHFV) (*1*). In recent years, nucleotide and amino acid sequence information has become available so that additional characterization of these viruses is possible, including further analysis of relationships among members of the genus. Full-length sequence data are now available for CCHFV, Hazara virus (HAZV) and Dugbe virus (DUGV), and partial sequences are available for many other members of the

genus. CCHFV, which ranges from sub-Saharan Africa to western People's Republic of China, is currently the most well characterized member of the genus. DUGV, also well characterized, is commonly isolated in surveillance studies conducted in Africa and appears to be endemic in most of the drier parts of this continent. DUGV is transmitted by ticks to vertebrates, including humans, and causes a mild febrile illness and thrombocytopenia (*2*).

In a recent survey of ticks infesting market livestock in Nairobi, Kenya, we identified 26 isolates of DUGV and additionally obtained several isolates of a virus that was identified as a nairovirus related most closely to DUGV (*3*). We report further characterization of the K611 isolate of this virus, including the full-length genome. Our findings suggest that this is a new virus in the genus *Nairovirus*, and we propose that it be designated Kupe virus (Kupe is the Kiswahili word for tick).

Materials and Methods

Isolates of viruses were obtained from pools of ticks collected at abattoirs in Nairobi, Kenya, as described (*3*). The K611 isolate used in this study was obtained from a pool of *Amblyomma gemma* ticks in October 1999.

Characterization of Viruses in Cell Culture and Mice

Growth of Kupe virus and DUGV was tested in Vero (African green monkey kidney), LLC-MK$_2$ (rhesus monkey kidney), BHK (baby hamster kidney), SW-13 (human adrenal cortex carcinoma), HeLa (human cervical adenocarcinoma), HUH-7 (human hepatocarcinoma), and C6/36 (*Aedes albopictus* mosquito) cells in culture. Growth kinetics of the 2 viruses were compared in a 13-day growth curve in which cells were infected at a multiplicity of infection of 0.01 and aliquots removed daily. Virus titers were assayed on Vero cell monolayers in

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6-well plates by using a published double-overlay method (*4*). Second overlays containing neutral red were added at 6-days postinfection.

Nucleic Acid Sequencing

Viruses to be sequenced were amplified in Vero cells, and viral RNA was extracted from cell culture supernatant by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Reverse transcription–PCR was conducted by using the Titan One Tube Reverse Transcription–PCR system (Roche, Indianapolis, IN, USA). Amplified products were purified by agarose gel electrophoresis, and DNA fragments were extracted by using the MinElute Gel Extraction Kit (QIAGEN). Purified DNA fragments were sequenced by using the BigDye 3.1 kit (PE Applied Biosystems, Foster City, CA, USA) and analyzed by using a model 3130 automated sequencer (PE Applied Biosystems). Both strands of the DNA were sequenced.

The full-length genome of Kupe virus isolate K611 was sequenced, beginning with fragments amplified by Nairobi sheep disease virus (NSDV)–specific primers or DUGV-specific primers from each segment. Full-length sequence was obtained by using a previously described method of primer walking and the 5′/3′ Rapid Amplification of cDNA Ends (RACE) Kit (Roche), which was used to determine the sequence of the segment ends (*5*). Fragments of the S (nt 413–916), M (nt 408–2372), and L (nt 6656–8185) segments from other Kupe virus isolates were also sequenced for comparison (*3*). Additionally, fragments of the S, M, and L segments from isolates of DUGV collected in 1999 from the Nairobi abattoirs were sequenced by using primers designed from the published sequence of DUGV (*3*).

Genome Characterization and Comparison with Other Viruses

The nucleotide sequence of each segment of the Kupe virus genome was analyzed for open reading frames (ORFs) by using the EditSeq module of Lasergene (DNAS-TAR, Inc., Madison, WI, USA) and translated into deduced amino acid sequence. Identification of protein motifs and potential sites for glycosylation was accomplished by using Prosite (http://ca.expasy.org/prosite), psi-BLAST and CDS-BLAST (www.ncbi.nlm.nih.gov/BLAST), NetOGlyc 3.1, and MOTIFS in the Wisconsin Package version 11.1.2 (*6,7*). Nucleotide and amino acid sequences were compared with DUGV, CCHFV, NSDV, and HAZV sequences. GenBank accession numbers for sequences used in this study are listed in Table 1 or in the text below. Sequence alignments were performed by using the PILEUP and GAP programs in the Wisconsin Package. Sequence identities were calculated by using the GAP program (Wisconsin Package) or MegAlign (Lasergene; DNASTAR, Inc.). Phylogenetic analysis of alignments was conducted by using the maximum parsimony method with 500 bootstrap replicates in MEGA, version 3.1 (www.megasoftware.net).

Results

Viruses were isolated from pools of ticks collected from livestock driven to market at 2 abattoirs in Nairobi, Kenya, as described (*3*). Several isolates made from pools of *A. gemma* and *Rhipicephalus pulchellus* ticks collected on 4 days during the fall of 1999 were identified as similar to DUGV on the basis of nucleotide sequence of a fragment of the S segment genomic RNA. This virus has been designated Kupe virus.

Growth kinetics of Kupe virus and DUGV were compared in 7 cell types (Figure 1). Neither virus replicated

Genome segment	Virus	Strain	GenBank nucleotide accession no.	GenBank amino acid accession no.
Small	Dugbe	ArD44313	AF434161	AAL73396
	Dugbe	KT281/75	AF434165	AAL73400
	Dugbe	IbAr1792	AF434164	AAL73399
	Dugbe	IbH11480	AF434163	AAL73398
	Dugbe	ArD16095	AF434162	AAL73397
	Nairobi sheep disease	RV082	AF504294	AAM33324
	Hazara	JC280	M86624	AAA43842
	Crimean-Congo hemorrhagic fever	IbAr10200	U88410	AAB48501
	Kupe	K611	EU257626	NA
Medium	Dugbe	ArD44313	M94133	AAA42974
	Hazara	JC280	DQ813514	ABH07417
	Crimean-Congo hemorrhagic fever	IbAr10200	AF467768	AAM48106
	Kupe	K611	EU257627	NA
Large	Dugbe	ArD44313	U15018	AAB18834
	Hazara	JC280	DQ076419	AAZ38668
	Crimean-Congo hemorrhagic fever	IbAr10200	AY389508	AAQ90157
	Kupe	K611	EU257628	NA

Table 1. Virus sequences used in phylogenetic comparisons*

*NA, not available.
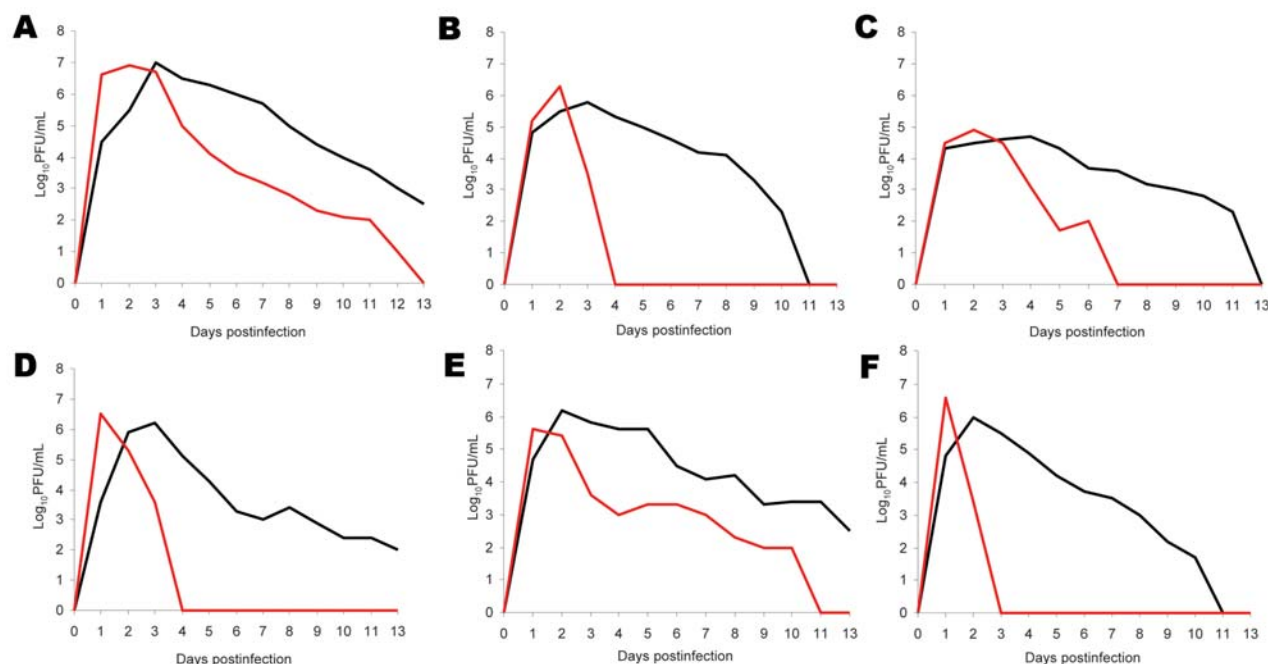
Figure 1. Growth of Dugbe (black lines) and Kupe (red lines) viruses in A) Vero (African green monkey kidney), B) LLC-MK₂ (rhesus monkey kidney), C) BHK (baby hamster kidney), D) SW-13 (human adrenal cortex carcinoma), E) HeLa (human cervical adenocarcinoma), and F) HUH-7 (human hepatocarcinoma) cells in culture.

in C6/36 mosquito cells. Kupe virus and DUGV replicated in all mammalian cell types tested, and maximum titers were observed 1–2 or 2–4 days postinfection, respectively. The Kupe virus titer increased more rapidly than the DUGV titers and achieved peak titers 1–2 days earlier. The subsequent decrease in titer was also more rapid (Figure 1). In all mammalian cell types except BHK cells, we observed earlier appearance of cytopathic effects (CPE) in Kupe virus–infected cells; CPE progressed more rapidly in DUGV-infected BHK cells. However, in all but LLC-MK₂ cells, Kupe virus caused greater overall destruction of the cell monolayer by the end of the growth curve experiment. In Vero cell plaque assays, DUGV plaques were slower to form than those caused by Kupe virus, although plaque morphology of the 2 viruses was similar (2–4 mm in diameter).

Genome Analysis

The 3 genomic RNA segments of Kupe virus, isolate K611, were completely sequenced, ORFs were identified, and deduced amino acid sequences were determined. Similar to other viruses in this family, the ends of each RNA segment contain a conserved sequence, the terminal 9 nt of which are identical to those found in all segments of DUGV, CCHFV, and HAZV and in the S segment of NSDV (sequence of other NSDV segments not available). The S segment of Kupe virus has 1,694 nt, an ORF of 483 aa, and 5′ and 3′ noncoding regions (NCRs) of 49 nt and 193 nt,

respectively. The DUGV S segment has 1,716 nt, 5′ and 3′ NCRs of 51 nt and 213 nt, and an ORF of 483 aa (8,9).

The Kupe virus M segment RNA has 4,818 nt and contains 1 ORF flanked by 5′ and 3′ NCRs of 47 nt and 121 nt, respectively. The DUGV M segment has 4,888 nt and its 5′ and 3′ NCRs are 47 nt and 185 nt, respectively (9). As observed in other nairoviruses, the Kupe virus M ORF, which has 1,549 aa, is longer than those of other members of *Bunyaviridae* (9,10). The Kupe virus M ORF contains 8 potential sites for N-linked glycosylation (N-gly); the DUGV M ORF contains 10 potential sites (Table 2). Kupe virus contains a unique potential N-gly site in the Gn and

Table 2. Potential N-linked glycosylation sites in the medium segment of Dugbe and Kupe viruses

Amino acid location*		Region†
Dugbe virus	Kupe virus	
25	16	Mucin-like, variable
30	–	Mucin-like, variable
80	–	Mucin-like, variable
142	140	Upstream of Gn
413	414	Gn
–	612	Gn
827	829	Unknown
848	–	Gc precursor
1201	1203	Gc
1258	–	Gc
1420	1421	Gc
—	1514	Gc

*Amino acid location in the translated open reading frame.
†Gn and Gc are glycoproteins.

Gc glycoprotein regions (aa 612 and aa 1514) and was missing potential sites found at aa 30, 80, 848, and 1258 in DUGV. Further analysis is necessary to determine which of the potential N-gly sites are used in DUGV and Kupe virus proteins. DUGV and Kupe virus M segment ORFs contain a highly variable, mucin-like region near the amino terminus, as described for the genome of CCHFV (9,11). This ≈100-aa region in DUGV and Kupe virus is shorter than the 243–248-aa region identified in CCHFV, but this region in both viruses contains similarly high amino acid sequence variability, increased frequency of serine, threonine, and proline residues, and more highly predicted O-linked glycosylation than for the rest of the ORF.

Previous studies of CCHFV and DUGV suggest that precursors of Gn and Gc glycoproteins are produced and then post-translationally cleaved to form mature glycoproteins. Potential tetrapeptide cleavage sites for SKI-1/S1P protease or a related protease have been identified immediately upstream of the N-termini of the CCHFV (RRLL[519]– Gn, RKPL[1040]– Gc) and DUGV (RKLL[374]– Gn, RKLL[896]– Gc[predicted]) glycoproteins; similar peptides are found in the Kupe virus ORF (RRIL[375] and RRLL[898]) (11–13). Additionally, a furin-like cleavage recognition motif (RSKR[247]) has been identified in CCHFV upstream of the amino terminus of Gn that has been shown to produce an additional glycoprotein; however, DUGV and Kupe virus do not share this motif (14). They contain an additional SKI-1/S1P-like cleavage motif in this region (DUGV–RRII[204]; Kupe virus– RRIL[202]).

As reported for DUGV and CCHFV, the length of the L segment RNA (12,330 nt) and ORF (4,050 aa) of Kupe virus is almost twice that of other bunyaviruses (15,16). The L RNA contains a 5′ NCR of 40 nt and a 3′ NCR of 137 nt; the 5′ and 3′ NCRs of DUGV are 40 and 104 nt, respectively. The Kupe virus ORF aa sequence shows a high degree of homology to that of DUGV, with the exception of a highly variable region (Kupe virus aa 755–896) that shows low homology (24.8%) and in which the DUGV sequence is 14 aa shorter than Kupe virus (42 nt deletion in DUGV

relative to Kupe virus). In this same region, a 92-nt deletion has been shown in CCHFV relative to DUGV, and a similar deletion is observed in HAZV (17). All conserved motifs in the RNA-dependent RNA polymerase (RDRP) module (region 3), as well as other conserved domains upstream and downstream of the polymerase module (regions 1, 2, and 4), were conserved in the Kupe virus ORF, as shown in DUGV and CCHFV (16). Kupe virus L segment ORF also contains several protein motifs previously identified in DUGV and CCHFV, including an ovarian tumor–like cysteine protease domain, a DNA topoisomerase–like domain (aa 76–94), and a C2H2-type zinc finger motif (aa 608–631) (17,18). However, Kupe virus ORF did not contain the leucine zipper motif identified in CCHFV and DUGV.

Phylogenetic Analysis

Nucleotide and deduced amino acid sequences of Kupe virus segments were compared with sequences from other nairoviruses available in GenBank and with partial sequences of DUGV isolates obtained in the 1999 Kenya survey in which Kupe virus was isolated (Tables 3–6) (3). Comparison of full-length S segment sequences showed 68.8%–69.4% nt and 74.9%–75.5% aa sequence identity between Kupe virus and 5 strains of DUGV. Identities among the 5 DUGV strain sequences were nt 90.9%– 99.4% and aa 98.1%–99.8%. Pairwise, full-length S segment nucleotide and amino acid identities among DUGV, CCHFV, NSDV, and HAZV ranged from 59.0%–64.1% and 55.3%–63.2%, respectively (see Table 3 for specific pairwise identities). A 428-nt fragment of the S segment, corresponding to Kupe S nt 44–471, was also sequenced from 26 DUGV isolates obtained during the 1999 abattoir survey (GenBank accession nos. FJ422213–FJ422238) and compared with available DUGV sequences from GenBank (Table 1) and Kupe virus. Results of these comparisons are shown in Table 6. Nucleotide and amino acid sequence identities among 5 Kupe virus isolates for a 504-nt fragment (nt 413–916) of the S segment were 95.0%–98.4% and 98.8%–100.0%, respectively (GenBank accession nos.

Table 3. Pairwise comparison of full-length nucleotide and amino acid sequences of the small segment of Kupe virus with other nairoviruses*

Virus	Kupe	Dugbe ArD44313	Dugbe ArD16095	Dugbe KT281/75	Dugbe IbH11480	Dugbe IbAr1792	NSDV	HAZV	CCHFV
Kupe		69.3	69.4	69.4	68.8	69.1	65.1	60.4	61.2
Dugbe ArD44313	75.2		99.3	91.1	98.9	99.1	63.6	60.0	59.6
Dugbe ArD16095	75.2	99.4		91.7	99.2	99.2	64.1	59.9	59.6
Dugbe KT281/75	74.9	98.1	98.3		91.0	90.9	63.3	59.1	59.0
Dugbe IbH11480	74.9	99.6	99.4	98.6		99.4	63.7	59.6	60.3
Dugbe IbAr1792	75.5	99.8	99.6	98.3	99.8		63.8	59.6	60.1
NSDV	64.0	59.9	60.1	59.5	59.9	60.1		63.5	63.1
HAZV	57.6	55.7	55.7	55.3	55.7	55.9	63.2		60.4
CCHFV	57.5	56.4	56.2	56.4	56.0	56.2	62.7	59.5	

*Nucleotide identity (%) is shown above the diagonal, and amino acid identity (%) is shown below the diagonal. NSDV, Nairobi sheep disease virus; HAZV, Hazara virus; CCHFV, Crimean-Congo hemorrhagic fever virus.

Table 4. Pairwise comparison of full-length nucleotide and amino acid sequences of the medium segment of Kupe virus with other nairoviruses*

Virus	Kupe	Dugbe ArD44313	HAZV	CCHFV
Kupe		61.9	54.7	52.1
Dugbe ArD44313	57.0		53.7	52.5
HAZV	47.7	44.4		50.8
CCHFV	43.0	38.3	41.4	

*Nucleotide identity (%) is shown above the diagonal, and amino acid identity (%) is shown below the diagonal. HAZV, Hazara virus; CCHFV, Crimean-Congo hemorrhagic fever virus.

EU257626, EU816906–EU816909). Results of phylogenetic analysis of the full-length S segment amino acid sequence alignment is shown in Figure 2, panel A. Kupe virus is shown as most closely related to DUGV, although it is distinct from the clade containing the 5 DUGV strains.

Full-length M segment sequences are available for only 3 of the known nairoviruses: DUGV (strain ArD 44313), HAZV, and CCHFV. Comparison of these viruses with Kupe virus M segment sequence showed 61.9%, 54.7%, and 52.1% nt identity and 57.0%, 47.7%, and 43.0% aa identity, respectively (Table 4). Additionally, a 308-nt fragment (Kupe M segment, nt 2181–2488) was sequenced from 25 DUGV isolates obtained in Kenya in 1999 (GenBank accession nos. FJ422239–FJ422263) and compared with DUGV ArD44313 and Kupe virus. Results of these comparisons are shown in Table 6. Sequence identities between 5 Kupe virus isolates for a 1,965-nt fragment of the M segment (nt 408–2372) were 90.9%–98.8% for nt and 96.0%–99.4% for aa (GenBank accession nos. EU257627, EU816902–EU816905). Phylogenetic analysis of full-length M segment amino acid sequences resulted in a tree with topology similar to that of the S segment tree (Figure 2, panel B).

Full-length L segment sequences are available only for DUGV (strain ArD 44313), HAZV, and CCHFV. Comparison of these sequences with Kupe virus sequence showed 77.4%, 62.8%, and 61.5% nt identity and 89.0%, 66.3%, and 63.7% aa identity, respectively (Table 5). As expected from this data, phylogenetic analysis of full-length L segment aa sequence resulted in a tree showing Kupe virus more closely related to Dugbe virus than in the S or M segment trees (Figure 2, panel C).

Nucleotide and amino acid sequence comparisons of a 441-nt fragment of the highly conserved L segment RDRP catalytic core domain (Kupe virus nt 6986–7426) were also made between Kupe virus and sequences of 14 other viruses representing 7 groups of the *Nairovirus* genus published by Honig et al. (*19*). A phylogenetic tree derived from the amino acid alignment of these sequences shows Kupe virus most closely related to DUGV (82.8% nt identity/95.9% aa identity), NSDV (74.9%/92.5%), CCHFV (71.9%/88.4%), and HAZV (71.7%/87.8%) (Figure 3). An additional 603-nt

L fragment alignment overlapping the RDRP core domain (Kupe virus nt 7292–7894) included sequences from 26 DUGV isolates obtained in Kenya in 1999 (GenBank accession nos. EU359010–EU359035), DUGV ArD 44313, and Kupe virus. Results of these comparisons are shown in Table 6. Sequence identities among 5 Kupe virus isolates for this fragment were nt 91.2%–100.0% and aa 98.5%–100.0% (GenBank accession nos. EU257628, EU816898–EU816901).

Discussion

Although little genetic information is available for most viruses in the genus *Nairovirus*, current classification of the diverse group of viruses in the genus is in relative agreement with available genetic analyses (*19,20*). Genetic information is useful in identifying emerging viruses and in analysis of relationships between viruses, especially given the segmented nature of the nairovirus genome, which can lead to generation of new viruses by segment reassortment (*21*). Within the genus, however, limited species and strain comparisons are available, making the definition of a genetic classification criteria difficult, and the segmented nature of the genome confounds the analysis. These findings are shown by a recent in-depth genetic analysis of CCHFV strains that demonstrated a high degree of genomic plasticity and RNA segment reassortment among virus strains studied (*22*).

Detailed study of the complete genome of 13 geographically and temporally diverse strains of CCHFV demonstrated nt/aa sequence identities of 80%/92%, 69%/73%, and 78%/90% for the S, M and L segments, respectively (*22*). Similarly, comparison of published full-length S segment sequences from 5 strains of DUGV isolated in Senegal, Nigeria, and Kenya between 1964 (IbAr1792) and 1985 (ArD443143) demonstrated sequence identities >90% at the nucleotide and amino acid levels (Table 3). Likewise, >89% identities were observed when a fragment of S segment sequence from these 5 strains was compared with 26 DUGV isolates from the 1999 Kenya abattoir survey (Table 3). S segment sequence identity between Kupe virus and DUGV falls well below identities observed among strains of either DUGV (Tables 3, 6) or CCHFV and is closer to that

Table 5. Pairwise comparison of full-length nucleotide and amino acid sequences of the large segment of Kupe virus with other nairoviruses*

Virus	Kupe	Dugbe ArD44313	HAZV	CCHFV
Kupe		77.4	62.8	61.5
Dugbe ArD44313	89.0		63.4	62.1
HAZV	66.3	66.1		62.3
CCHFV	63.7	63.4	64.0	

*Nucleotide identity (%) is shown above the diagonal, and amino acid identity (%) is shown below the diagonal. HAZV, Hazara virus; CCHFV, Crimean-Congo hemorrhagic fever virus.

Table 6. Nucleotide and amino acid sequence comparisons between fragments of Kupe and Dugbe viruses*

Segment and virus	Virus		
Small (428 nt)	Kupe	Dugbe, Kenya, 1999	Other Dugbe†
Kupe		68.8–70.9	69.2–70.9
Dugbe, 1999, Kenya	67.6–69.7		89.3–97.9
Other Dugbe†	69.0	95.8–100.0	
Medium (308 nt)	Kupe	Dugbe, Kenya, 1999	Dugbe ArD44313
Kupe		63.9–65.2	65.8
Dugbe, 1999, Kenya	61.8–64.7		86.8–92.3
Dugbe ArD44313	63.7	93.1–98.0	
Large (603 nt)	Kupe	Dugbe, Kenya, 1999	Dugbe ArD44313
Kupe		81.8–82.4	81.3
Dugbe, 1999, Kenya	94.5–96.0		91.0–92.0
Dugbe ArD44313	94.5	96.0–98.0	

*Nucleotide identity (%) is shown above the diagonal, and amino acid identity (%) is shown below the diagonal.
†Dugbe viruses listed in Table 1.

observed in S segment sequence comparisons among DUGV, CCHFV, NSDV, and HAZV (Table 3) (*22*).

Although comparison of full-length M segment sequence among multiple DUGV strains is not possible because of lack of available sequence information, sequence identities for comparison of a fragment of the M segment of DUGV ArD44313 and the 26 isolates obtained in Kenya in 1999 were >86% for nt and >93% for aa. In contrast, identities observed between Kupe virus and the DUGV sequences were considerably lower and, similar to the S segment sequence, were closer to identities observed among DUGV, CCHFV, and HAZV. In addition, differences in

the number and positions of potential N-gly sites in the M segment ORF between DUGV and Kupe virus suggest substantial differences between these viruses.

Comparison of Kupe virus L segment sequences was inconclusive in determining its relationship to DUGV. Again, because of lack of available sequence information, comparison of multiple full-length DUGV strains is not possible at this time; comparison of a fragment of the L segment between the Kenya DUGV isolates and DUGV ArD44313 showed identities >91%. The relatively high full-length L segment nt/aa sequence identities of 77.4%/89.0% observed between Kupe virus and DUGV strain ArD 44313 are simi-
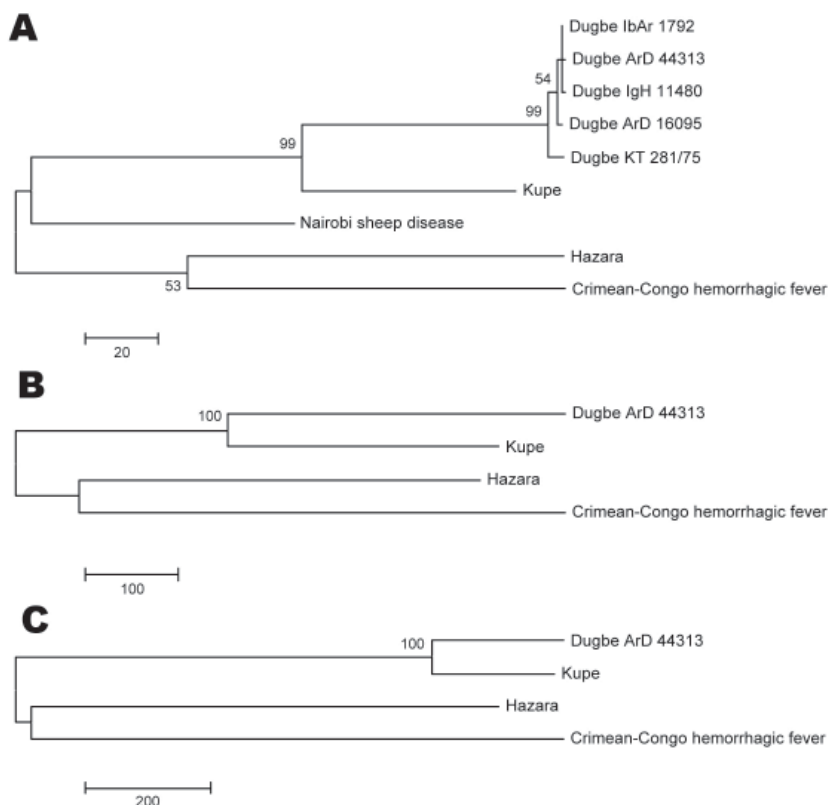
Figure 2. Phylogenetic trees produced by using maximum-parsimony analysis with 500 bootstrap replicates on alignments of full-length amino acid sequences of the A) small segment, B) medium segment, and C) large segment of Kupe virus with other available full-length nairovirus sequences. Scale bars indicate branch length and bootstrap values >50% are shown above branches.
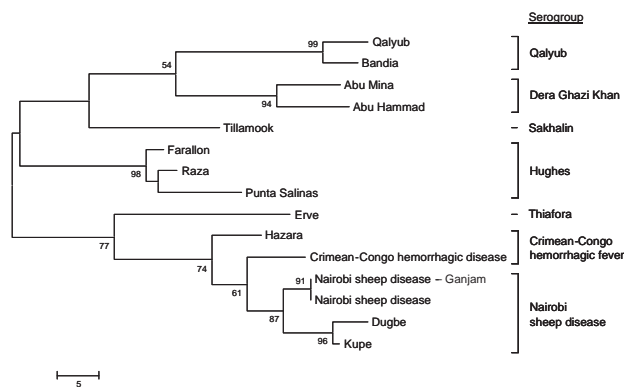
Figure 3. Phylogenetic tree produced by using maximum parsimony analysis with 500 bootstrap replicates on amino acid alignment of nairovirus large segment fragment (147-aa sequence translated from 441-nt sequence). Scale bar indicates branch length, and bootstrap values >50% are shown above branches.

lar to identity levels reported among full-length L segment comparisons of CCHFV strains. This finding suggests that the L segment of Kupe virus may have been acquired from DUGV by reassortment. However, identities observed for the highly conserved 603-nt L segment fragment between Kupe virus and DUGV ArD44313 were somewhat lower compared with identities between DUGV ArD44313 and other Kenya DUGV isolates. These lower identities, combined with differences observed in the L segment variable region (Kupe virus aa 755–896), suggest otherwise.

Little is known about the ecology of Kupe virus other than its isolation from ticks infesting cattle. DUGV has been reportedly isolated from several tick species, including *A. gemma* and *R. pulchellus*, the species from which Kupe virus was isolated (*19,23,24*). In the 1999 Kenya abattoir survey, DUGV was isolated from 4 species of ticks, *A. variegatum*, *A. gemma*, *A. lepidum*, and *R. pulchellus* (*3*). Although ≈1,000 specimens each of *A. variegatum* and *A. lepidum* were collected and tested in that study, no isolates of Kupe virus were found in those species, which suggested that vector hosts for DUGV and Kupe virus may differ (*3*). Specific vector competence studies will be needed to resolve this point. The pathogenesis, if any, of Kupe virus in mammals is unknown.

Kupe virus and DUGV were observed to replicate and cause CPE in a variety of cultured mammalian cell types. Kupe virus was observed to have a more rapid increase and subsequent decrease in viral titer, an earlier onset of visible CPE, and greater destruction of the cell monolayer in most of the mammalian cells tested. These findings show that this virus is more virulent than DUGV in the mammalian cells tested.

Taxonomic classification of viruses is an evolving discipline that in early years was based primarily on mor-

phologic characters. More recently, better classification has been obtained by using antigenic relationships and information gained from genetic characteristics. The International Committee on Taxonomy of Viruses has defined a virus species as "a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche" (*25*). This definition and its use in virus classification has been the subject of much discussion in the literature, and its application to newly described viruses is often difficult because of incomplete descriptive information about the new virus and other viruses in the group to which it is related (*26,27*).

For Kupe virus, nucleotide and amino acid sequence variation between the S and M segments of Kupe virus and DUGV, or any other genetically characterized nairovirus, was greater than expected between strains of a single virus in the genus *Nairovirus*. We also noted differences in other genetic characteristics between Kupe virus and DUGV, including M segment N-gly sites, L segment variable region, and NCR length variations. This evidence, combined with increased virulence of Kupe virus in cultured mammalian cells and potential differences in vector hosts, shows that Kupe virus is substantially different from, although closely related to, DUGV and is a new virus in the genus *Nairovirus*. However, further studies are necessary to determine the hosts, vectors, and geographic range of Kupe virus along with its virulence as a human or animal pathogen. Such information will aid in appropriate classification of this new virus.

Ms Crabtree is a researcher at the Centers for Disease Control and Prevention in Fort Collins, Colorado. Her research interest is the molecular biology of arboviruses.

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Oseltamivir-Resistant Influenza Viruses A (H1N1), Norway, 2007–08

Siri H. Hauge, Susanne Dudman, Katrine Borgen, Angie Lackenby, and Olav Hungnes

In Norway in January 2008, unprecedented levels of oseltamivir resistance were found in 12 of 16 influenza viruses A (H1N1) tested. To investigate the epidemiologic and clinical characteristics of these viruses, we used sequence analysis to test all available subtype H1N1 viruses from the 2007–08 season for resistance. Questionnaires from physicians provided information on predisposing diseases, oseltamivir use, symptoms, and complications. Clinical data were obtained for 265 patients. In total, 183 (67.3%) of 272 viruses were oseltamivir resistant. Resistance was not associated with prior use of antiviral drugs. Symptoms and hospitalization rates did not differ for patients infected with a resistant or a susceptible virus. Oseltamivir-resistant influenza viruses A (H1N1) did not show diminished capability to spread in the absence of selective pressure. The ability of these viruses to sustain their fitness and spread among persons should be considered when shaping future strategies for treating and preventing seasonal and pandemic influenza.

Seasonal influenza, caused by influenza A subtypes H3N2 and H1N1 and influenza B viruses, occurs as annual epidemics. Although vaccination remains the primary measure for prevention, antiviral drugs are available for prevention and treatment of influenza. The influenza virus neuraminidase inhibitors zanamivir and oseltamivir were introduced into clinical practice in various parts of the world from 1999 through 2002 (1). Oseltamivir limits replication of both influenza A and B viruses (1). In most European countries, neuraminidase inhibitors are not widely used to treat seasonal influenza, but they are being stockpiled in many countries as part of their pandemic influenza preparedness. In Norway, oseltamivir is registered for prophylactic and therapeutic use in persons >1 year of age; however, it is not available without a prescription and it is rarely prescribed (2).

Until 2007, resistance against neuraminidase inhibitors was rarely observed (1,3,4). Nevertheless, to better understand the potential for development of resistance against neuraminidase inhibitors, surveillance of antiviral susceptibility in influenza virus in Europe has been ongoing since 2004 (5). As part of the World Health Organization (WHO) Global Influenza Surveillance Network, the national influenza centers in Europe submit influenza viruses to the WHO Influenza Collaborating Centre in the United Kingdom each influenza season. Within the framework of the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL), these viruses are also tested for drug susceptibility at the Health Protection Agency in London.

In mid-January 2008, antiviral susceptibility testing (enzyme inhibition assays) of the first shipment of influenza viruses from Norway for the 2007–08 season showed an unusually large proportion (5/7) of influenza viruses A (H1N1) with high-level resistance to oseltamivir. In subsequent days, testing of additional viruses from Norway at the Norwegian national influenza center and at the Health Protection Agency confirmed the high proportion of oseltamivir resistance. This unexpected and unprecedented discovery had possible public health implications of international concern. On January 25, 2008, the Norwegian Institute of Public Health notified WHO of these findings under the International Health Regulations (6) and notified the European Commission through the Early Warning and Response System. The Institute also informed hospitals and physicians in Norway about a possible lack of therapeutic effect when treating patients with oseltamivir. By the end of January, oseltamivir-resistant viruses had been reported from several European countries (7).

The oseltamivir-resistance trait is caused by a previously described point mutation in the virus neuraminidase gene (histidine to tyrosine at position 275 of the N1 neuramini-

dase, commonly referred to as H274Y in N2 numbering), which is known to confer high-level resistance to oseltamivir while retaining susceptibility to zanamivir (*8*). Influenza viruses A (H1N1) carrying the H274Y mutation have reduced ability to replicate and transmit efficiently when compared with parental, susceptible virus, but the clinical implications of infection with these viruses have been largely unknown (*9*). Consequently, we undertook studies to determine whether the emergence and spread of the resistant viruses were associated with exposure to oseltamivir, whether resistant viruses would continue to circulate in similar proportions into the epidemic phase of the season, and whether the new resistant viruses differed from their susceptible counterparts in their ability to cause disease. To do so, we tested all influenza viruses A (H1N1) available from the 2007–08 outbreak for oseltamivir susceptibility. We furthermore enhanced surveillance by collecting an extended set of data regarding clinical symptoms, complications, and prior exposure to oseltamivir for all laboratory-verified cases of influenza viruses A (H1N1) infection.

Methods

The influenza viruses A (H1N1) included in this study were obtained from the sentinel and nonsentinel collaborators as part of routine national virologic influenza surveillance. From all 19 counties, 71 sentinel practices collect samples from patients with influenza-like illness and send them to the national influenza center for diagnostic testing. From all parts of the country, 15 medical microbiology laboratories submit materials containing influenza A or B materials (original specimens, nucleic acid preparations from original specimens, or viral isolates) to the national influenza center for further characterization. Most of these samples originate from primary care clinics; the rest, from hospitals.

Viruses confirmed as influenza A (H1), by either reverse transcription–PCR (RT-PCR) or virus isolation in MDCK cells and subsequent subtyping by immunofluorescence, were included in the study. In-country susceptibility testing was performed by detecting the H274Y mutation by sequence analysis, through either a pyrosequencing assay targeting the single relevant point mutation (*10*) or through full- or partial-length cycle sequencing of the coding region for the viral neuraminidase. These analyses were mostly performed on RNA prepared from the original patient specimen. A large proportion of the isolated viruses were sent to the WHO Collaborative Centre for Influenza Research and Reference in the National Institute of Medical Research, Mill Hill, UK, for further characterization. Within the framework of VIRGIL, these viruses were forwarded to the Health Protection Agency for phenotypic antiviral susceptibility testing and more extensive genotypic analyses. To determine neuraminidase susceptibility, assays to determine the drug concentration that provides 50% inhibition (IC_{50}) were performed by using the fluorescent substrate methylumbelliferyl N-acetylneuraminic acid based on the method described by Wetherall et al. (*11*) with minor modifications.

Relative quantitative data on virus shedding, i.e., virus RNA content in the patient specimens, were obtained through a real-time RT-PCR targeting a conserved part of the matrix protein (M1) gene of influenza A virus. Two microliters of nucleic acid prepared from specimens (MagNApure LC Total Nucleic Acid Isolation Kit; Roche Diagnostics, Mannheim, Germany) was added to a 23-µL reaction mixture containing 0.3 µM forward primer M52c (5′-CTT CTA ACC GAG GTC GAA ACG-3′); 0.3 µM reverse primer M149r (5′-CTT GTC TTT AGC CAT TCC ATG AG-3′); 0.15 µM probe M93c (FAM-5′ CCG TCA GGC CCC CTC AAA GCC GA 3′-Black Hole Quencher 1); and 5× QIAGEN OneStep RT-PCR buffer, dNTP mixture, and enzyme mixture according to the manufacturer's instructions (QIAGEN OneStep RT PCR Kit; QIAGEN, Hilden, Germany). Forward primer and probe sequences were as described by Fouchier et al. (*12*), and the reverse primer was designed by Tom Øystein Jonassen (Akershus University Hospital, Lørenskog, Norway). Reactions were run in a Corbett Rotorgene RG-3000 or RG-6000 thermocycler (Corbett Research Pty Ltd, Sydney, New South Wales, Australia) with the following cycling conditions: reverse trancription for 30 min at 50°C, then 15 min at 95°C, followed by 50 cycles at 95°C for 10 sec, 54°C for 30 sec, and 72°C for 20 sec.

Participants and Study Design

We included all patients with a diagnosis of influenza virus A (H1N1) infection made by national influenza center during the 2007–08 influenza season. For the 72 patients who received this diagnosis before the end of January 2008, data were collected retrospectively. From February on, data were collected as soon as possible after laboratory confirmation of influenza virus A (H1N1) infection. Structured questionnaires returned from consulting physicians provided auxiliary information about clinical signs and symptoms, complications, predisposing diseases for severe outcome of influenza (diabetes, cardiac disease, lung disease, and immunodeficiency), use of oseltamivir, and influenza vaccination status. If the questionnaire was not returned by mail within 3 weeks, a reminder call was made. When available, relevant clinical information on the original referral sample form was used to supplement the data from the written questionnaire. The consulting physician, usually the primary care physician, was not informed about the result of the susceptibility testing when the information was collected. Information for the first 12 patients infected with a resistant virus was collected from the consulting physicians by telephone.

Statistical Analysis

Data from the questionnaires and selected laboratory testing outcomes were merged, checked for quality, and analyzed by using Stata version 9.0 (StataCorp LP, College Station, TX, USA). We used the Fisher exact test to compare the proportions of possible confounders among those infected with a resistant and a susceptible virus. To estimate the association between exposure (resistant virus infection) and outcome (subsequent clinical findings and complications), we calculated crude risk ratios (RRs) and 95% confidence intervals (CIs). We used binomial regression to calculate RRs adjusted for possible confounders. For each variable, we used the number of respondents as the denominator, except for predisposing disease, for which missing values were coded as "no."

Results

The overall influenza activity in Norway was low in 2007–08 compared with that of previous years. Virologic surveillance showed most influenza virus A and 95% of subtyped viruses to be subtype H1N1 (13). From the sentinel practices, the national influenza center received 229 specimens for influenza testing. Of the 108 that were positive for influenza virus, 61 were type A, subtype H1N1, and most of the rest were type B. In total, 297 patients had an influenza virus A (H1N1) infection confirmed by the national influenza center in Norway from week 47 in 2007 until the end of week 20 in 2008. We obtained a resistance profile for 272 of the 297 viruses. We could not determine the resistance profile for the remaining 25 because of low virus content and consequently excluded them from analysis.

A total of 196 viral isolates were available (133 carried the resistance mutation); of these, 113 (79 with the resistant genotype) were reference tested by the VIRGIL laboratory. Phenotypic and genotypic reference analysis results agreed completely with the in-country genotypic testing results; all mutant viruses showed large reductions in susceptibility to oseltamivir when compared with non-H274Y viruses (IC_{50} 260–2,161 nM, mean 673 nM, for the 274Y mutant and 0.4–5.6 nM, mean 2.6 nM, for the nonmutant viruses). No evidence of mixed genotype or phenotype was observed. In phylogenetic analysis of the H1 gene, all viruses tested grouped together in subclade 2B (Figure 1). In the phylogenetic tree, the resistant viruses from Norway all formed a single branch that was distinct, but closely related, to the susceptible viruses from Norway.

Of the 272 influenza viruses A (H1N1), 183 (67.3%) were oseltamivir resistant (Table 1). The proportion of resistant viruses did not differ between samples from sentinel 67.9% (38/56) and nonsentinel 67.1% (145/216) practices and persisted throughout the season (Figure 2). No difference in virus shedding, as quantified by real-time RT-PCR

of available patient specimens, was observed between susceptible and resistant viruses (Figure 3). From the original sample form, we obtained demographic information for all 272 patients. Returned questionnaires provided information for 265 patients (97.4%), but the response rate on individual questions varied. Of the 272 patients infected
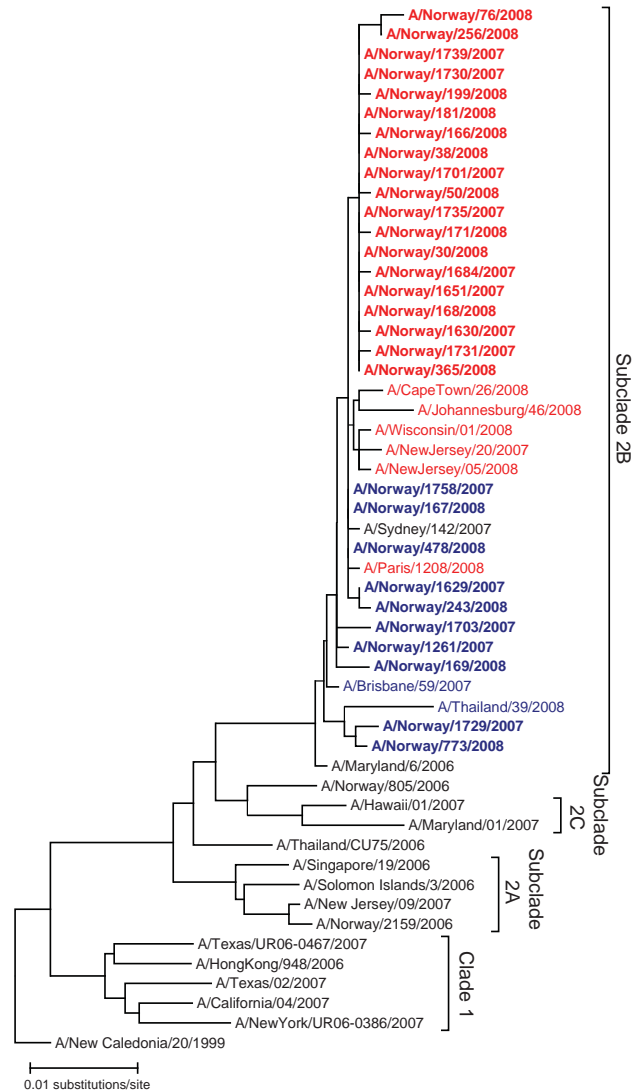
Figure 1. Phylogenetic reconstruction of the H1 genes of influenza viruses A (H1N1) in Norway, 2007–08 season. The analysis was performed on an alignment spanning positions 84–1054 of viral RNA segment 4. Pairwise distances were calculated by using the Kimura 2-parameter model with a transition:transversion ratio of 2.0; the phylogenetic tree was constructed by the neighbor-joining method, as implemented in the programs DNADIST and NEIGHBOR in the PHYLIP package (14,15). Published sequences were obtained from the Influenza Sequence Database, Los Alamos National Laboratory (16). **Boldface** indicates viruses from the 2007–08 influenza season in Norway; red indicates oseltamivir-resistant viruses; blue, susceptible viruses. New sequences presented in this analysis have been deposited in GenBank (accession nos. CY036664–CY036694).

Table 1. Proportion of oseltamivir-resistant and oseltamivir-susceptible influenza viruses A (H1N1), 2007–08 influenza season, Norway

Sample source	Total no. samples	No. (%) resistant samples	No. (%) susceptible samples
All	272	183 (67.3)	89 (32.7)
Type of practice			
Sentinel	56	38 (67.8)	18 (32.1)
Nonsentinel	216	145 (67.1)	71 (32.9)
Patient gender			
Male	132	85 (64.4)	47 (35.6)
Female	140	98 (70.0)	42 (30.0)
Patient age group, y			
0–4	45	27 (60.0)	18 (40.0)
5–14	45	25 (55.6)	20 (44.4)
15–24	31	20 (64.5)	11 (35.5)
25–59	138	102 (73.9)	36 (26.1)
60–99	13	9 (69.2)	4 (30.8)
Patient with predisposing disease			
Diabetes	10	9 (90.0)	1 (10.0)
Lung disease	11	8 (72.7)	3 (27.3)
Cardiac disease	5	2 (40.0)	3 (60.0)
Immunodeficiency	5	3 (60.0)	2 (40.0)
Any	27	20 (74.1)	7 (25.9)

with influenza viruses A (H1N1), 132 (48.5%) were male (Table 1), and slightly more than half (50.7%) were 29–64 years of age (median 27 years, range 2 months–71 years); median ages of those infected with a resistant and a susceptible virus were 31 and 21 years, respectively. The highest proportion of resistant virus infection was found for those 25–59 years of age (102/138, 73.9%) and differed significantly from the proportion for only those 5–14 years of age (25/45, 55.6%) (Fisher exact p = 0.03). We obtained influenza viruses A (H1N1) from 18/19 counties (Figure 4).The oseltamivir resistance proportion was ≥80% in 8 counties in southern Norway, compared with 63.5% in the rest of the country (Fisher exact p = 0.001).

Information about use of antiviral drugs was obtained for 237 patients. No patients had received antiviral treatment in the 14 days before the onset of symptoms, and none had been in close contact with others known to have used antiviral drugs. Oseltamivir was received after sampling by

Figure 2. Oseltamivir-resistant (n = 183) and oseltamivir-susceptible (n = 89) influenza viruses A (H1N1) in the 2007–08 influenza season in Norway, by week of sampling.

7 patients, 5 of whom were infected with an oseltamivir-resistant virus. Of 225 patients, 9 had traveled abroad in the week before symptom onset; 4 were infected with a resistant virus. Of all 272 patients, 2 had been vaccinated against influenza and were both infected with a resistant virus.

We received information about predisposing disease for 213 patients. Having a predisposing disease more than doubled the risk for complications (RR 2.5, 95% CI 1.2–5.4) but was not clearly associated with being infected with a resistant virus (RR 1.4, 95% CI 0.6–3.2). Information about clinical symptoms was obtained for 252/272 patients; most frequently reported were fever (229/242) and dry cough (182/218). Resistant virus infection was not associated with any particular symptom (Table 2). Of 241 patients, 58 (24.1%) had ≥1 complications recorded, but no difference was observed between those infected with a resistant virus and those infected with a susceptible virus (Table 2). Bronchitis and pneumonia were the most frequent complications, reported for 22 and 17 patients, respectively. The age of the 17 patients who had pneumonia ranged from 8 months to 65 years (mean 29 years): 2 (12.5%) were 0–4 years of age, 5 (31.3%) were 5–14 years of age, 2 (12.5%) were 15–24 years of age, 4 (25.0%) were 25–59 years of age, and 3 (18.8%) were >59 years of age. Of the 17 patients with pneumonia, 15 were infected with a resistant virus. The attack rates of pneumonia and of sinusitis were higher for those infected with a resistant virus than for those infected with a susceptible virus, although the risk ratios were not statistically significant after adjusting for age, gender, and predisposing disease (pneumonia RR 3.2, 95% CI 0.7–13.7; sinusitis RR 1.7, 95% CI 0.4–7.5) (Table 2). Of 264 patients, 45 had been hospitalized, 28 and 17 in-
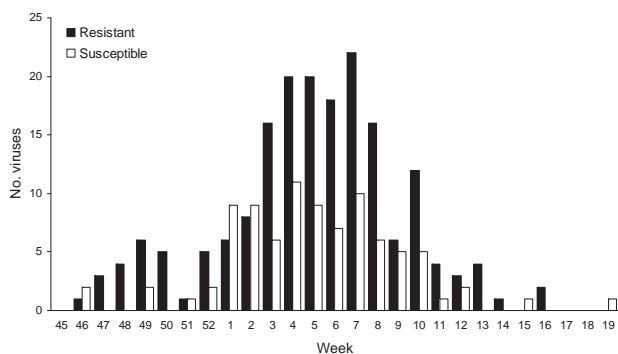
Table 2. Reported associations for patients infected with oseltamivir-resistant or oseltamivir-susceptible influenza virus A (H1N1), 2007–08 influenza season, Norway*

Association	Resistant, n = 183		Susceptible, n = 89		Crude associations	Adjusted associations†
	Attack rate, %	No. responses	Attack rate, %	No. responses	RR (95% CI)	RR (95% CI)
Sign or symptom						
Productive cough	38.4	125	31.9	47	1.2 (0.8–1.9)	
Fever	94.4	162	95.0	80	1.0 (0.9–1.1)	
Myalgia	72.9	129	73.3	60	1.0 (0.8–1.2)	
Dry cough	82.1	145	86.3	73	1.0 (0.8–1.1)	
Headache	63.4	131	67.2	58	0.9 (0.8–1.2)	
Sore throat	57.5	134	67.2	58	0.9 (0.7–1.1)	
Runny nose	62.2	127	66.1	56	0.9 (0.8–1.2)	
Complication						
Pneumonia	9.2	153	2.9	69	3.2 (0.7–13.5)	3.2 (0.7–13.7)
Sinusitis	6.2	145	3.0	67	2.1 (0.5–9.4)	1.7 (0.4–7.5)
Otitis media	4.8	145	4.4	69	1.1 (0.3–4.2)	1.3 (0.4–4.8)
Bronchitis	8.7	149	11.8	76	0.7 (0.3–1.7)	0.8 (0.4–1.8)
Any	24.4	164	22.1	77	1.1 (0.7–1.8)	1.1 (0.7–1.8)
Hospitalization	15.8	177	19.5	87	0.8 (0.5–1.4)	0.8 (0.5–1.3)

*RR, risk ratio; CI, confidence interval.
†RR adjusted for age, gender, and predisposing disease.

fected with a resistant and a susceptible virus, respectively. No deaths were reported for patients included in the study.

Discussion

During the 2007–08 influenza season in the Northern Hemisphere, widespread circulation of oseltamivir-resistant influenza viruses A (H1N1) was observed. Percentage of resistant viruses circulating in different countries varied markedly; the highest proportion reported worldwide (67%) was in Norway (*17,18*).

Our study did not show any association between oseltamivir use in Norway and emergence of the oseltamivir-resistant influenza viruses A (H1N1). Because only a minority of influenza cases are laboratory confirmed, oseltamivir use in nonsampled persons could have contributed to the development of resistance. However, for this suggestion to be plausible, use of oseltamivir would have to be widespread to exert substantial selective pressure on the viruses. Sales of oseltamivir in Norway have been low: 699 5-day regimens (0.15/1,000 population) were sold in 2004; 66,249 (14.4/1,000 population) in 2005; 33,573 (7.3/1,000 population) in 2006; and 4,686 (1.0/1,000 population) in 2007 (*2*). In countries where oseltamivir use has been high, e.g., Japan, the proportion of oseltamivir-resistant influenza viruses A (H1N1) reported during the 2007–08 season was low (*18*). Because influenza strains from Norway were genetically similar to resistant viruses that appeared just as early in several other European countries (A. Hay, pers. comm.), we consider it unlikely that the resistant variant originated in Norway. Conceivably, the initial emergence of a resistant virus could be associated with oseltamivir use elsewhere. Our data indicate that the viruses carrying this resistance mutation are fully capable of persistence and spread in the absence of selective pressure.

In Norway, the initially high proportion of resistant influenza viruses A (H1N1) was maintained throughout the entire 2007–08 influenza season; countrywide, 2 of 3 viruses
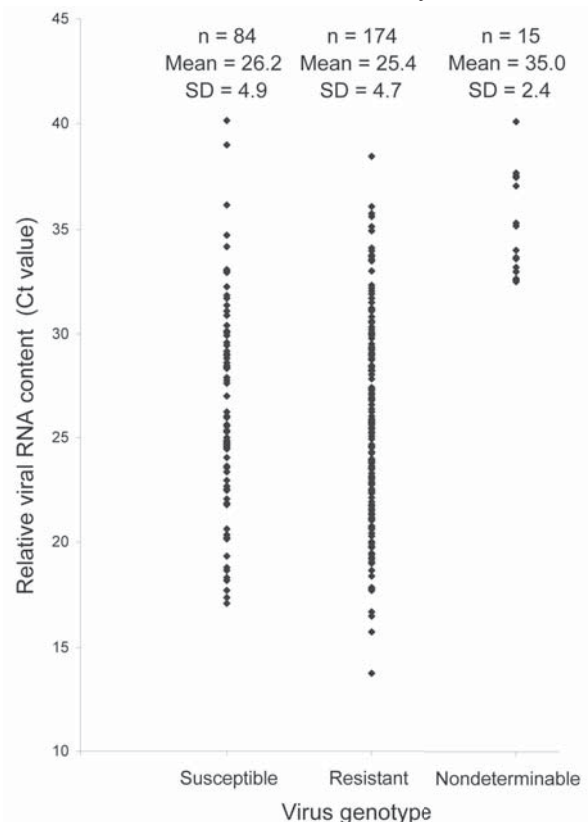
Figure 3. Comparison of virus shedding, measured as relative viral RNA content, in respiratory specimens taken from patients infected with oseltamivir-susceptible and oseltamivir-resistant influenza viruses A (H1N1), respectively, during the 2007–08 influenza season in Norway. Viral RNA content is expressed as the reverse-transcription–PCR cycle number (Ct) during which the fluorescence threshold was exceeded.

Figure 4. Proportion of oseltamivir-resistant influenza viruses A (H1N1) in the 2007–08 influenza season in Norway, by county of sampling. The total number of samples analyzed for each county is given inside each county.

were resistant. The reason for this exceptionally high resistance proportion is unknown. However, it likely reflects the proportion of resistant viruses introduced into Norway in the fall of 2007. Globally, the proportion of resistant influenza viruses A (H1N1) reported is highly variable between the different countries for which data are available (*18*). This large variation, apparently in the absence of oseltamivir selective pressure, suggests that a high level of randomness determined the frequency of resistance. In temperate countries, the influenza viruses are reintroduced each autumn after the absence of influenza during the summer. If only a limited number of viruses are reintroduced into each country and initiate virus circulation and outbreaks, the result will be considerable random variation in virus variant proportions between the different countries. Consistent with this result, almost all the characterized influenza viruses A (H1N1) in Norway could be assembled into a small number of genetically discernible groups (Figure 1). We propose that such randomness in virus introductions may be sufficient to explain the differences in the proportions of resistant viruses between the countries.

Conceivably, a difference in the antigenic characteristics of the resistant and susceptible viruses could have fa-

vored one virus over the other in the face of host population immunity. Such differences might contribute to different relative effect of the 2 viruses in different populations (e.g., countries) or subpopulations (e.g., age groups). However, the resistant and susceptible viruses were closely related and were not distinguishable in hemagglutination inhibition tests (*19*).

Overall, the observed clinical manifestations associated with influenza viruses A (H1N1) in this study were as expected for seasonal influenza. No differences were noted for virus shedding, primary symptoms, or overall complication and hospitalization rates caused by oseltamivir-resistant and -susceptible viruses. We did find, although not a statistically significant finding, that patients infected with a resistant virus appeared to be more likely than those infected with a susceptible virus to have pneumonia or sinusitis. Patients with more severe illness may be more likely to be sampled; however, the resistance pattern of the virus was not known by the physician at the time of sampling and reporting. We therefore believe that these findings are not influenced by selection bias. Adjusting for possible confounders (age, sex, and predisposing disease) did not change the results. Because of our limited sample size, the precision of our estimates is low, but they do indicate findings that warrant further investigation. Our data will also be analyzed with data from other European countries, and the findings may strengthen the conclusions about the clinical implications of oseltamivir-resistant influenza viruses A (H1N1) .

The future effect of resistant influenza viruses A (H1N1) is unpredictable. In Europe, the H1N1 subtype was predominant during the 2007–08 influenza season and, according to historical patterns, is unlikely to predominate during the 2008–09 influenza season. In the following 2008–09 season in the Northern Hemisphere, influenza viruses A (H1N1) may well predominate in areas where they had not recently been present in large numbers. Early reporting from the Southern Hemisphere 2008 influenza season indicates that detection of influenza virus A (H1N1) is low (*20*). However, in South Africa, oseltamivir resistance has been detected in 100% of influenza viruses A (H1N1) tested (*21*).

Whether oseltamivir-resistant viruses will persist beyond 2008 depends on several factors. First, their persistence will depend on the prevalence of resistant viruses in the populations that are the source of global influenza spread. Countries in East and Southeast Asia have been proposed as the most likely source for global dissemination of new influenza virus variants (*22*). The prevalence of resistant influenza viruses A (H1N1) in this region may therefore be more likely to influence future occurrence of these viruses than the prevalence in Europe; resistance monitoring thus needs to be global. Second, changes in re-

cent influenza viruses A (H1N1) may have provided a genetic background that permits H274Y mutants to replicate and transmit. Previous studies have concluded that resistant viruses are less pathogenic and less transmissible than their susceptible counterparts (*9,23*). In contrast, however, reverse genetics–derived mutants (A/WSN/33 or PR8 backbone) had the same phenotype as wild type viruses in vitro and in vivo (*24,25*). A recent study on the enzymatic properties of the N1 neuraminidase of the resistant viruses from the 2007–08 season suggested some genetic background changes that could potentially be involved (*26*).

As long as such a postulated permissive genetic background is common, resistant mutants may arise anew in purely oseltamivir-susceptible influenza virus A (H1N1) populations. Identification of such predisposing genetic traits and monitoring of their occurrence in influenza viruses A (H1N1) and other influenza viruses should continue.

Similar resistance can arise in viruses other than the current human influenza viruses A (H1N1). Resistance in a more virulent influenza virus can have serious public health implications because of fewer therapeutic and prophylactic options, which may result in more persons being affected by influenza and more severe illness and death in those who become infected. Oseltamivir is a prime option for influenza treatment and prophylaxis and forms a substantial part of pandemic preparedness in many countries. The prevalence of oseltamivir-resistant viruses reported in Europe throughout the 2007–08 influenza season clearly shows that this resistant mutation is stable and that these viruses sustain their fitness and ability to spread among persons. These findings should be taken into consideration when shaping future strategies for treating and preventing seasonal and pandemic influenza.

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Epidemiology of *Vibrio parahaemolyticus* Outbreaks, Southern Chile

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Disease outbreaks caused by *Vibrio parahaemolyticus* in Puerto Montt, Chile, began in 2004 and reached a peak in 2005 at 3,600 clinical cases. Until 2006, every analyzed case was caused by the serovar O3:K6 pandemic strain. In the summer of 2007, only 475 cases were reported; 73% corresponded to the pandemic strain. This decrease was associated with a change in serotype of many pandemic isolates to O3:K59 and the emergence of new clinical strains. One of these strains, associated with 11% of the cases, was genotypically different from the pandemic strain but contained genes that were identical to those found on its pathogenicity island. These findings suggest that pathogenicity-related genes were laterally transferred from the pandemic strain to one of the different *V. parahaemolyticus* groups comprising the diverse and shifting bacterial population in shellfish in this region.

In 1998 in Antofagasta, Chile (23°39′S, 70°24′W), ≈300 human cases of infection with *Vibrio parahaemolyticus* caused by consumption of contaminated seafood were reported (*1*). Outbreaks have not been observed in this region since 1998. During 2004–2007, ≈7,000 cases were reported farther south in Puerto Montt (41°29′S, 72°24′W) (*2–5*). However, outbreaks generally have been decreasing; there were ≈1,500 cases in 2004, 3,600 in 2005, 900 in 2006, and 475 in 2007 (*6*) (http://epi.minsal.cl/epi/html/Actualidad/Vibrio.htm).

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Until 2006, ≈100% of the cases analyzed were caused by a clonal group originally observed in Southeast Asia in 1996 (*4,5,7*). This group was known as the *V. parahaemolyticus* pandemic strain because it had reached coastal environments worldwide and caused outbreaks. It belongs to the O3:K6 serovar, although at least 21 serovariants have emerged since 1996 (*8*). These serovariants also have specific sequences corresponding to genes such as *toxRS/new* (*9*), open reading frame 8 (*orf8*) (*10*), and *tdh*, but lack others such as *trh*, which is found in other pathogenic strains.

Genome sequencing of the RIMD2210633 pandemic strain showed that it has 2 sets of gene clusters that encode the type III secretion system (TTSS) apparatus (*11*). This apparatus is used by several gram-negative pathogenic bacteria to secrete and translocate virulence factor proteins into the cytosol of eukaryotic cells (*12*). TTSS1 is involved in cytotoxicity against HeLa cells, and TTSS2 is involved in enterotoxic activity in a rabbit ileal loop test (*13*). The first cluster is located on the large chromosome, and the second is located on the small chromosome. The second cluster contains 2 copies of the *tdh* gene and is located on a pathogenicity island (a discrete genetic unit that contains genes responsible for pathogenicity and virulence) probably obtained by recent lateral transfer (*11*). TTSS2 has been found only in strains showing β-type hemolysis on a specialized blood agar medium known as Wagatsuma agar (*11*). This hemolysis is called the Kanagawa phenomenon and is considered a useful marker for identification of pathogenic strains. Recently, TTSS genes related to the TTSS2 cluster were reported in clinical and environmental non-O1, non-O139 *V. cholerae* strains (*14*).

The clonal nature of the pandemic *V. parahaemolyticus* isolates was ascertained by the similarity of patterns

obtained by genome restriction fragment length polymorphism–pulsed-field gel electrophoresis (*15*), arbitrarily primed PCR (*7,9*), direct genome restriction enzyme analysis (DGREA) (*4*), and multilocus sequence typing (MLST) (*16,17*). However, the pandemic strain is a minor fraction of a diverse and shifting *V. parahaemolyticus* population found in shellfish in Puerto Montt (*2*). In an effort to understand the epidemiology of these outbreaks, we studied *V. parahaemolyticus* isolates obtained from human cases and shellfish during the summer of 2007. Our results indicate replacement of the O3:K6 pandemic strain by new serotype O3:K59 and new pathogenic *V. parahaemolyticus* groups. The decrease in the number of clinical cases may have been caused by diminution of the *V. parahaemolyticus* O3:K6 pandemic group in regional seafood.

Methods

Strains

V. parahaemolyticus RIMD 2210633 (VpKX) and RIMD 2210086 (VpI) were obtained from the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. The Chilean environmental strains, identified as PMA with a number according to origin and year of isolation, were obtained from shellfish samples obtained during outbreaks from 2004 though 2007. Most strains have been described (*4*). PMC38.7, PMC60.7, PMC53.7, and PMC75.7 are isolates from clinical samples obtained in 2007. Each of these isolates corresponds to the isolate type of the 23 groups differentiated by DGREA as described (*4*) and reported in this article.

Analysis

Samples of clinical cases and shellfish were obtained and analyzed as described (*4*). Isolation, growth, and characterization of isolates, including their DGREA patterns, were conducted as described (*4,5*). Each DGREA pattern found in 2007 was compared with those for previous years. When similarities in patterns were observed, their identity was checked by comparing patterns obtained in the same electrophoretic analysis.

PCR assays were performed by using ≈10 ng of total bacterial DNA per reaction tube. Amplification conditions were those previously reported for *tlh*, *tdh*, and *trh* (*18*), orf8 (*19*), and *toxRS/new* (*9*) genes. T3SS2 genes (VPA1335, VPA1338, VPA1339, VPA1341, VPA1342, VPA1346, VPA1349, VPA1354, VPA1355, VPA1362, and VPA1367) were amplified by using primers reported for a microarray assay at 61ºC by Meador et al. (*20*). Genes VPA1321 and VPA1376, which are located at the extremes of the pathogenicity island, were amplified by using primers designed using the Primer3 program (http://primer3.sourceforge.net). Sequences of these primers were VPA1321f: 5′-TGACATGCACGGCAATAGAT-3′, VPA1321r: 5-ACAGAGTTGGTTTCGCAGGT-3′, VPA1376 f: 5′-CATCGAGCGATCTTTCACAA-3′, and VPA1376r: 5′-ACCGGTTTCCAACCTTCTCT-3′. Housekeeping genes for MLST were amplified by using primers for *V. parahaemolyticus* (*17*) and from the MLST website (http://pubmlst.org/vparahaemolyticus) developed by Keith Jolley (University of Oxford, Oxford, UK) (*21*).

PCR products were purified by using either the Wizard SV Gel or PCR Clean-Up Systems (Promega, Madison, WI, USA) and sequenced in both directions by Macrogen (Seoul, South Korea) or by McLAB (South San Francisco, CA, USA) by using forward and reverse amplification primers or primers M13F and M13R (MLST loci). DNA sequences were analyzed individually and manually assembled. Alignments and sequence similarities were obtained by using BioEdit software (*22*). Sequences obtained were deposited in GenBank under accession nos. EU185060–EU185092.

Amplification and sequencing of the variable region of the 16S rRNA (*rrs*) between nucleotides 357 and 518 (*Escherichia coli* numbering) were performed as described (*23*). This analysis consisted of separation of *rrs* alleles in PMC38.7 by pulsed-field gel electrophoresis and PCR amplification of the variable region in excised bands as described (*24*).

Results

V. parahaemolyticus Associated with Human Cases in 2007

V. parahaemolyticus isolates from 37 human case-patients with diarrhea from the summer of 2007 in the Puerto Montt region were analyzed and grouped according to serotype, presence of genetic markers (*orf8*, *toxRS/new*, *tlh*, *tdh*, *trh*), and distinctiveness of their DGREA patterns (Table 1; Figure 1). One isolate from each patient was characterized. On the basis of genetic markers and DGREA pattern, isolates from 27 patients corresponded to the pandemic clonal group. However, 40% of the 20 serotyped strains of this group contained a K_{59} capsular antigen instead of the characteristic K_6 antigen, and 25% cross-reacted with antisera for K_6 and K_{59} antigens. Another difference from strains of previous years was that the relative number of cases associated with the pandemic strain (73%) was lower than the 100% observed in previous summers (*4*). Isolates from the other 27% of patients with clinical cases lacked the characteristic markers of the pandemic strains, i.e., *orf8* and *toxRS/new*, and had 4 new DGREA groups. One of these groups contained 4 *tdh*-positive isolates (11% of cases). A second group contained 4 isolates positive for *tdh* and *trh* genes. The other 2 groups contained 1 isolate each, and both were negative for these 2 markers (Table 1). One

Table 1. Properties of *Vibrio parahaemolyticus* clinical isolates from Puerto Montt, Chile, summer 2007*

Isolate	*tlh*	*tdh*	*trh*	*orf8*	ToxRS/new	Serotype	DGREA group	MLST ST†
PMC50.7, 51.7, 41.7, 72.7, 1.7, 15.7, 16.7	+	+	–	+	+	O3:K6	VpKX	3
PMC55.7, 56.7, 58.7, 28.7, 44.7, 73.7, 14.7, 18.7	+	+	–	+	+	O3:K59	VpKX	ND
PMC29.7, 42.7, 70.7, 11.7, 19.7	+	+	–	+	+	O3:K6,59	VpKX	ND
PMC**59.7**, **63.7**, **64.7**, **65.7**, **66.7**, **20.7**, **22.7**	+	+	–	+	+	ND	VpKX	ND
PMC38.7, **47.7**, 57.7, 68.7	+	+	–	–	–	O10:K20	38.7	63
PMC60.7, **25.7**, **26.7**, 27.7	+	+	+	–	–	O1:KUT	60.7	64
PMC53.7	+	–	–	–	–	O3:K59	1.5	28
PMC75.7	+	–	–	–	–	O1:KUT	75.7	65

*Isolates with an undetermined serotype are in **boldface**. *orf*, open reading frame; DGREA, direct genome restriction enzyme analysis; MLST, multilocus sequence typing; ST, sequence type; ND, not determined.
†Sequences are available from http://pubmlst.org/vparahaemolyticus.

isolate chosen as the type strain of each group was typed by MLST (*17*). MLST sequence type corresponded with groups determined by other analyzed genetic properties (Table 1).

Characterization of Nonpandemic Strains

Strains positive for *tdh,* other than the pandemic strain, had not been isolated from patients with clinical cases in Puerto Montt during the 3 previous summer outbreaks. It has been reported that this gene may be spread by insertion sequence–like elements (*25,26*). The possibility that *tdh* found in nonpandemic strains was derived from the pandemic strain was explored. PCR amplicons of the *tdh* gene of the 2 nonpandemic groups was sequenced in isolates designated as type strains for each group: PMC60.7 for the group containing *tdh* and *trh* and PMC38.7 for the group containing only *tdh*. The amplicon of isolate PMC60.7 had an identical sequence to that reported for *tdhA* except for 1 nucleotide (*11*). Conversely, PMC38.7 had an identical sequence to that expected for a mixture of the 2 *tdh* genes in VpKX. These 2 genes differ slightly and the mixture of their PCR products should show polymorphisms in specific sites (*11*).

This observation suggested the presence of *tdhS* and *tdhA* genes in PMC38.7 with identical sequences to those found in the pandemic strain. Because these 2 genes are located close to each end of the pathogenicity island in chromosome 2 of the pandemic *V. parahaemolyticus* (*11*), the presence of the entire island was explored by PCR amplification of 11 genes of TTSS2 located in the island and of genes VPA1321 and VPA1376 located at the extremes of the island near *tdhA* and *tdhS*, respectively. Each tested gene was found in the PMC38.7 strain, and sequences of their PCR products were identical to those reported for the pandemic strain genes, except for 1 nucleotide in VPA1342. Serotyping of PMC38.7 indicated an O10:K20 serovar. Because MC38.7 is genetically different from the pandemic strain (Table 1, Figure 1), the high degree of homology of these genes suggested that the entire pathogenicity island had recently been transferred from the pandemic strain.

PMC38.7 also differs from the pandemic strain and most clinical isolates by the presence of intragenomic heterogeneity among its multiple 16S rRNA genes, a feature seldom observed in clinical isolates but frequently observed among environmental isolates. This finding is probably caused by lateral transfer of *rrs* (*23*). Three *rrs* genes, with sequences corresponding to *V. parahaemolyticus* groups VpD1-B4, ATA65-B2, and VpKX-AB (*23*), were observed in PMC38.7.

V. parahaemolyticus Associated with Shellfish

There are a large number of *V. parahaemolyticus* strains in the environment in the Puerto Montt region. Only the pandemic strain was isolated from clinical samples before and during the summer of 2006, but 20 different
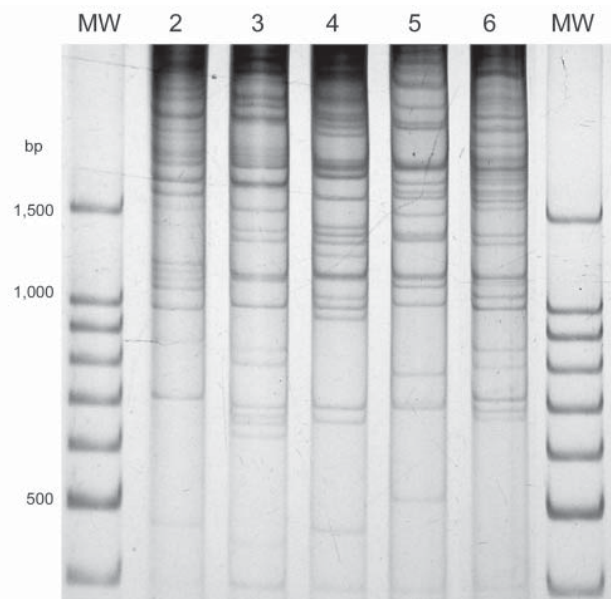

Figure. 1. Direct genome restriction enzyme analysis with *Nae*I of clinical isolates of *Vibrio parahaemolyticus* representative of the 5 patterns observed during the outbreaks in Puerto Montt, Chile, January and February, 2007. Lanes MW, 100-bp size ladder; lane 2, PMC38.7; lane 3, PMC60.7; lane 4, PMC53.7; lane 5, PMC75.7; lane 6, VpKX. (O3:K6 pandemic isolate).

Table 2. Properties of *Vibrio parahaemolyticus* isolates from shellfish from Puerto Montt, Chile, summer 2007*

Isolate	*tlh*	*tdh*	*trh*	*orf8*	ToxRS/new	DGREA group	MLST ST†
PMA4.7, 5.7, 6.7, 7.7, 8.7,15.7, 16.7, 17.7, 19.7, 20.7, 22.7, 23.7, 24.7, 25.7, 26.7, 27.7, 28.7, 33.7, 37.7, 41.7, 42.7, 43.7, 47.7, 48.7, 49.7	+	–	–	ND	ND	34.6	ND
PMA9.7, 10.7, 12.7, 14.7, 18.7, 38.7	+	–	–	ND	ND	118	10
PMA1.7, 2.7, 3.7	+	–	–	ND	ND	1.7	ND
PMA11.7, 13.7	+	–	–	ND	ND	11.7	ND
PMA21.7	+	–	–	ND	ND	21.7	ND

*orf, open reading frame; DGREA, direct genome restriction enzyme analysis; MLST, multilocus sequence typing; ST, sequence type; ND, not determined.
†Sequences are available from http://pubmlst.org/vparahaemolyticus.

strains were isolated from shellfish during that period (*2*). Characterization of 52 isolates from 20 shellfish samples during the summer of 2007 indicated ≥5 DGREA groups; 3 of them were not previously observed (Table 2; Figure 2). This finding increases the number of different strains found in shellfish of the region to 23. Figure 3 shows the number of shellfish samples containing each of the 23 DGREA groups found during the past 4 years, including the summer of 2007 (*2*; this study). Only 4 of the 20 shellfish samples examined produced *tdh*-positive enrichment cultures, which is indicative of pathogenic strains. However, no *tdh*-positive isolates were obtained from these enrichments after plating on thiosulfate citrate bile salts sucrose agar.

T3SS2 Genes in Other Nonpandemic *V. parahaemolyticus* Strains

Because the presence of TTSS2 genes is not exclusive to the pandemic strain (*20*), their occurrence in the other environmental and clinical *V. parahaemolyticus* DGREA groups found in Puerto Montt was explored. We analyzed

Figure 2. Direct genome restriction enzyme analysis with *Nae*I of *Vibrio parahaemolyticus* isolates from shellfish collected in Puerto Montt, Chile, summer 2007. Gel shows representative strains for every observed pattern. Patterns of groups observed in previous years are next to the type isolate of that group. Lanes MW, 100-bp size ladder; lane 2, PMA4.7; lane 3, 34.6; lane 4, PMA9.7; lane 5, 118; lane 6, PMA1.7; lane 7, PMA11.7; lane 8, PMA21.7.

by PCR amplification 20 strains corresponding to 18 environmental DGREA groups (PMA79, 112, 118, 189, 337, 339, 3316, 1.5, 19.5, 22.5, 27.5, 13.6, 34.6, 36.6, 40.6, 1.7, 11.7, and 21.7) and 1 strain from each of 3 new clinical groups (PMC60.7, 53.7, and 75.7) found in Puerto Montt in 2007 for the VPA1335 gene (found in T3SS2). Among these strains, only PMA339, isolated from shellfish in the summer of 2004, was positive. PCR amplification of PMA339 for the other genes in the pathogenicity island showed positive reactions for all the genes tested in PMC38.7. Nevertheless, sequences of amplicons showed strong differences from those found in PMC38.7 and those reported for the pandemic strain genome. Similarity ranged from 99.4% for VPA1362 to 93.8% for VPA1346; the average for all genes tested was 97.7%. These differences indicate a much larger evolutionary distance between the T3SS2 genes in PMA339 and the pandemic strain than between PMC38.7 and the pandemic strain.

Discussion

The epidemiology of outbreaks caused by *V. parahaemolyticus* in the Puerto Montt region is changing. The number of clinical cases caused by the pandemic strain has decreased, accompanied by a change of serotype from O3:K6 to O3:K59. The changing serotype of the pandemic strain has been recently reviewed by Nair et al. (*8*). These authors showed that the more recent serotypes do not have the propensity for increasing hospital admissions observed with O3:K6, and some type of change in the epidemic process seems to be evident. Genes for the biosynthesis of capsular polysaccharides, which are major antigens (K) of *V. parahaemolyticus*, are probably encoded in a gene cluster characterized by variability that may occur through lateral gene transfer (*27*). Isolates reacting with antisera for K_6 and K_{59} antigens may have changed part of the K epitopes reacting with the commercial polyclonal antiserum.

The percentage of clinical cases caused by the pandemic strain decreased from 100% in 2006 to 73% in 2007. Four clinical strains, not previously observed, emerged in 2007. Among these, 1 group representing 11% of the clinical cases, with type strain PMC38.7, may have recently received the genes on the pathogenicity island of the pan-
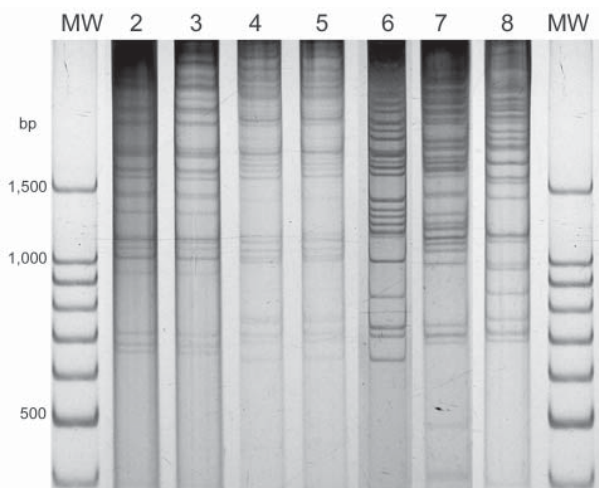
Figure 3. Number of seafood samples containing *Vibrio parahaemolyticus* corresponding to different direct genome restriction enzyme analysis (DGREA) groups observed in Puerto Montt, Chile, each summer, 2004–2007.

demic strains. Pathogenicity island genes identical to those in the pandemic strain in this bacterial group and differences in housekeeping genes and DGREA patterns are best explained by transfer of the pathogenicity island from the pandemic strain to an indigenous strain. The indigenous *V. parahaemolyticus* population in shellfish is diverse, and the predominant strains seem to change every year. A detailed examination of the putative genomic island in PMC38.7, its integration site, and its flanking regions, will probably help differentiate among possible mechanisms of DNA transfer.

The presence of TTSS2 genes is not exclusive of the pandemic and PMC38.7 strain; they were also found in an environmental isolate of *V. parahaemolyticus* (PMA339). However, PMA339 has not been observed among clinical isolates. TTSS2 genes have been found in other *V. parahaemolyticus* clinical strains (*20*); however, we identified them in environmental strains. On the basis of sequences obtained from their amplicons, TTSS2 genes in PMA339 seem to have independently evolved from the pandemic strain over a considerable time. Nevertheless, they are still more closely related to the TTSS2 of *V. parahaemolyticus* than to those recently found in non-O1 or non-O139 *V. cholerae* (*14*).

Little is known of the origin of the other 3 clinical groups (60.7, 1.5, and 75.7). Group 60.7 contains *tdh* and *trh*, but this *tdh* does not seem to be derived from the pandemic strain. Strains of groups 1.5 and 75.7 lack both pathogenicity-associated genes, but finding these isolates in patients is not unusual (*28*). PMC75.7, a clinical strain, contains a *recA* gene that is closely related to that of PMA339, the environmental isolate containing TTSS2 genes (*17* and http://pubmlst.org/vparahaemolyticus). However, in view of a recent report (*29*), one should consider that the 2 human isolates lacking *tdh* or *trh* genes may correspond to nonvirulent strains that proliferate during infection with a virulent strain.

The abundance and frequency of pandemic and non-pandemic *V. parahaemolyticus* in shellfish seem to have been less in 2007 than in previous years. In 2006, 10 of 20 shellfish samples were positive for *tdh* after enrichment; in 2007, only 4 of 20 samples were positive. PCR amplification of colonies obtained after plating the enrichment culture on thiosulfate citrate bile salts sucrose agar enabled identification of *tdh*-positive colonies in 6 samples in 2006; none could be identified by the same method in 2007. The observed decrease in outbreaks was probably caused by a decrease in raw seafood consumption as a result of a public health campaign and a decrease in the load of the highly virulent pandemic strain in shellfish. However, this tendency could change on the basis of dispersion and virulence of emerging pathogenic strains.

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Identification of Melioidosis Outbreak by Multilocus Variable Number Tandem Repeat Analysis

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Endemic melioidosis is caused by genetically diverse *Burkholderia pseudomallei* strains. However, clonal outbreaks (multiple cases caused by 1 strain) have occurred, such as from contaminated potable water. *B. pseudomallei* is designated a group B bioterrorism agent, which necessitates rapidly recognizing point-source outbreaks. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) can identify genetically related isolates, but results take several days to obtain. We developed a simplified 4-locus multilocus variable number tandem repeat analysis (MLVA-4) for rapid typing and compared results with PFGE and MLST for a large number of well-characterized *B. pseudomallei* isolates. MLVA-4 compared favorably with MLST and PFGE for the same isolates; it discriminated between 65 multilocus sequence types and showed relatedness between epidemiologically linked isolates from outbreak clusters and between isolates from individual patients. MLVA-4 can establish or refute that a clonal outbreak of melioidosis has occurred within 8 hours of receipt of bacterial strains.

Melioidosis is endemic in Southeast Asia and northern Australia (*1,2*). The reported incidence of melioidosis has been increasing within this region, and new foci and outbreaks of melioidosis are being described within this region and in distant locations such as Brazil (*3*) and New Caledonia (*4*). It remains unclear how much of this expansion of the global distribution boundaries is from recent spread of the causative bacterium, *Burkholderia pseudomallei*, and how much is from unmasking of disease after events such as the 2004 Asian tsunami (*5,6*). Molecular studies have shown considerable genetic diversity within *B. pseudomallei* (*1,2,7*). For instance, in northern Australia, isolates from patients are generally distinct from each other (*8*) unless there is a point-source outbreak, such as occurred in 2 episodes after *B. pseudomallei* contamination of community water supplies (*9,10*).

In disease-endemic regions, melioidosis case numbers surge in the monsoonal wet season, and individual cases are typically caused by different *B. pseudomallei* strains. However, under some circumstances, a series of cases can be caused by 1 strain, indicating that a clonal or point-source outbreak has possibly occurred and an urgent public health response may be required. Because several days are required to perform the currently available molecular typing methods of ribotyping, multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE), the ability to rapidly distinguish endemic infection from a clonal outbreak has not been possible. Furthermore, *B. pseudomallei* is classified as a group B bioterrorism agent by the US Centers for Disease Control and Prevention, and the implications of a possible deliberate release warrant the availability of a robust method to quickly ascertain if concomitant cases of melioidosis are caused by 1 bacterial strain.

We recently described using a BOX-PCR for rapid typing of *B. pseudomallei* (*11*). We have now adapted and simplified multilocus variable number tandem repeat (VNTR) analysis (MLVA) for rapid typing because this analysis potentially enables precise international strain comparisons. We have compared MLVA results on a wide range of well-characterized *B. pseudomallei* isolates with those for MLST and PFGE.

Methods

MLVA, PFGE, and MLST

U'Ren et al. initially described 32 VNTR loci for *B. pseudomallei* that had 7–28 alleles (*12*). Thirty of these VNTR markers were subsequently used for fine-scale analysis of 121 isolates of *B. pseudomallei* (*13*). Various combinations of markers were tested by MLVA; we chose 4 markers that were highly discriminatory, enabling single-run, 4-color analysis in a DNA sequencer. The 4 VNTR loci chosen were 2341, 389, 1788, and 933 (*12*). Table 1 shows the PCR primers used and the repeat region amplified for each locus. VNTR loci 2341 and 933 are from *B. pseudomallei* chromosome 1, and loci 389 and 1788 are from chromosome 2.

PCRs contained 0.88 U HotStarTaq DNA Polymerase (QIAGEN, Hilden, Germany) per reaction, 1× PCR buffer, 1.2 M Betaine, 3 mmol/L MgCl$_2$, 0.2 mmol/L deoxynucleoside triphosphates, 0.2 μM fluorescently labeled forward primer, 0.2 μM reverse primer, 1 μL template DNA (0.5 ng/μL), and double-distilled water to give a volume of 11 μL per reaction. Amplifications were conducted in Palm Cyclers (Corbett Research, Sydney, New South Wales, Australia). All PCRs underwent initial denaturation at 95°C for 5 min, then 34 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 30 s, followed by a final extension step of 72°C for 5 min and 15°C for 3 min.

PCR products of each colored primer (FAM, NED, PET, and VIC; Table 1) were then pooled. Pooled PCR products were diluted with 200 μL of double-distilled water, and 1.2 μL of PCR product was added to a mixture of a 1:6 ratio of Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and GeneScan 1200 LIZ (Applied Biosystems) fluorescently labeled size standard. PCR products were then electrophoretically separated by using a 3100xl DNA Sequencer (Applied Biosystems) and analyzed by using the ABI software program GeneMapper version 3.5

(Applied Biosystems). PFGE with *Spe*I and MLST were performed as described (*7,14*).

Data Analysis

For 4-locus multilocus VNTR analysis (MLVA-4), GeneMapper peak files were imported into BioNumerics version 4.61 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Relatedness of isolates was assessed by using a matrix of the pairwise differences of the 4 VNTR loci, with a dendrogram produced by using the unweighted pair group method with arithmetic averages (UPGMA).

For PFGE, gel images were analyzed with BioNumerics version 4.61. BioNumerics application modules used were Fingerprint Types and Comparison and Cluster Analysis modules. PFGE bands (150–700 kbp) were manually assigned on visual inspection. PFGE dendrograms were produced with Dice UPGMA with position tolerance settings of 0.5% optimization and 1.0% band position tolerance.

For MLST, alleles at each of the 7 previously described loci (*7*) were assigned for each isolate by comparing sequences to those at the *B. pseudomallei* MLST website (*15*). Following the standard MLST protocol, each allele was assigned a different allele number, and the allelic profile (string of 7 integers) was used to define the sequence type (ST) for that isolate. Allelic profiles of isolates were imported into BioNumerics version 4.61, and relatedness of isolates was displayed as a dendrogram by using the matrix of pairwise differences in the allelic profiles and UPGMA clustering.

B. pseudomallei Isolates

To assess the discriminatory power of MLVA-4, direct comparisons were made between the MLST dendrogram for 65 *B. pseudomallei* isolates, each representing a distinct ST, and the MLVA-4 dendrogram for these isolates. The 65 isolates were all from Australia and included human, animal, and environmental sources. There were 16 pairs of

Table 1. Characteristics of 4 VNTR loci used for identification of *Burkholderia pseudomallei**

Characteristic	VNTR loci			
	2341	1788	933	389
Color-labeled forward primer sequence (5′ → 3′)	FAMGGCTTCGCACC CGCCCCATTTCAGC	PETGCGCGGCGAGA ACGGCAAGAACGAA	NEDATGGTGGCGGC CGTCGGCGAAAACC	VICGTTACAAGC GCGGGTCGGCA AGAGGCTGAAA
Reverse primer sequence (5′ → 3′)	GCACCGGGCGCGGC GCACTCG	GAGCATCGGGTGGG CGGCGCGTATTGAT	GCTCGAATGGGTGT ACGAAGGGCCACGC TGATTC	GCCGGTGTTGA ACGAGTGGGTG GCGTAAGC
Repeat sequence (5′ → 3′)	TTCGTGCGC	GTCGTGCGATCCTG CT	CGGCGAGGGAAA	GACGAACC
Minimum size, bp† (no. repeats)	111 (2)	235 (4)	171 (3)	221 (1)
Maximum size, bp (no. repeats)	243 (17)	382 (13)	337 (17)	292 (10)
No. alleles	16	10	13	9
No. null alleles	–	–	2/65 STs	–

*VNTR, variable number tandem repeat; STs, sequence types.
†Error range in fragment sizing is ± 3 bp.

single-locus variants (SLVs; isolates sharing identical alleles at 6/7 loci by MLST).

To assess the ability of MLVA-4 to identify clonal clusters, direct comparisons were made between the PFGE dendrogram for 4 defined clonal groups and the MLVA-4 dendrogram for these isolates. Clonal cluster I and clonal cluster II consist of 8 and 7 isolates, respectively, from the tropical Northern Territory of Australia and were previously identified as clustering by PFGE (16). These 2 clonal clusters represent geographically linked but epidemiologically unrelated isolates from our prospective melioidosis studies in northern Australia. Clonal cluster III consists of 3 isolates of identical ST cultured from a detergent container implicated in an outbreak of melioidosis in Northern Territory involving 2 garage mechanics (14). Clonal cluster IV contains 6 isolates (5 from humans, 1 from water) from an outbreak of melioidosis in a remote indigenous community in Northern Territory. The outbreak was linked to contamination of the unchlorinated community water supply, with several deaths reported (10).

Finally, to assess the ability of MLVA-4 to link isolates from patients, we analyzed multiple isolates from 3 patients. Patient A had chronic pulmonary melioidosis, and 5 B. pseudomallei isolates were recovered over 22 months. Patient B had chronic pulmonary melioidosis, and 7 isolates were recovered over 6 years, including 2 isolates from this patient's water supply. Patient C died of melioidosis septicemia; 6 isolates were recovered over 14 days. To construct the dendrogram for these clinical isolates, we chose 6 unrelated isolates representing the diversity of Australian isolates seen with MLVA-4 (Table 2). These 6 isolates are indicated in Figure 1. This study was reviewed and approved by the Human Research Ethics Committee of the Northern Territory Department of Health and Community Services and the Menzies School of Health Research, Darwin, Northern Territory, Australia (approval 02/38).

Results

Table 1 shows size variation with calculated number of repeats and number of alleles for each of the 4 VNTR loci. Locus 933 showed null alleles for 2 of the 65 MLST STs.

Figure 1 shows the relationship between the 65 discrete MLST STs and the MLVA-4 for these isolates. MLVA-4 was able to discriminate between each ST. Relationships between STs seen on the MLST dendrogram were generally not preserved with MLVA-4. This is expected because VNTRs change too rapidly and too few loci were used to compensate for homoplasy at individual loci and to provide phylogenetic content to the assay. However, strains that were closely related by MLST (SLVs) could in some cases be seen to be related by using MVLA-4 (Figure 1).

Figure 2 shows results for the 24 isolates in the cluster study, with 4 additional unlinked isolates, each from a different ST included for comparison. There was generally excellent agreement between PFGE and MLVA-4 for each of the 4 clonal clusters. PFGE clonal clusters I (MLST ST 132) and II (ST 109), each containing epidemiologically unrelated strains, also clustered on MLVA-4, with the exception of isolate MSHR1429, which by MLVA-4 was located outside its cluster group. The detergent cluster III (ST 123) was indistinguishable by MLVA-4, and the community outbreak strains in cluster IV (ST 125, ST 126) separated into 2 closely linked MLVA-4 patterns, 1 of which included the isolate from the community water supply (MSHR491, ST 126).

Figure 3 shows MLVA-4 results for the 3 patients. Isolates from patient A (ST 243) and B (ST 131) with chronic pulmonary melioidosis each had closely linked MLVA-4 results with a suggestion of fine-scale differentiation over the years of infection. The 2 water supply isolates from patient B were identical to 5 of her clinical isolates. The 6 clinical isolates from patient C, who had fatal melioidosis, were identical by MLVA-4, including isolates from blood and sputum.

Discussion

Ribotyping was the first method widely used for typing B. pseudomallei (17), followed by PFGE. To date, PFGE has been considered the standard method for investigating potential point-source outbreaks of bacterial infections. We have previously used PFGE to link case clusters of melioidosis to water supply contamination (10) and to

Table 2. Fragment size and repeat copy number (MLVA-4 code) for 6 *Burkholderia pseudomallei* strains used as MLVA-4 standards*

| | VNTR loci | | | | | | | |
| | 2341 | | 389 | | 1788 | | 933 | |
Strain	Size, bp†	Repeat copy no.	Size, bp†	Repeat copy no.	Size, bp†	Repeat copy no.	Size, bp†	Repeat copy no.
MSHR978	189.85	11	236.19	8	265.63	6	254.8	10
MSHR1822	190.25	11	245.02	9	282.56	7	290.55	13
MSHR114	145.49	6	252.32	10	298.4	8	242.57	9
MSHR1641	154.5	7	236.22	8	315.39	9	230.81	8
MSHR1153	127.93	4	236.24	8	298.51	8	194.96	5
MSHR1123	172.34	9	260.28	11	331.48	10	218.91	7

*MLVA-4, 4-locus multilocus variable number tandem repeat analysis.
†Error range in fragment sizing is ± 3 bp.
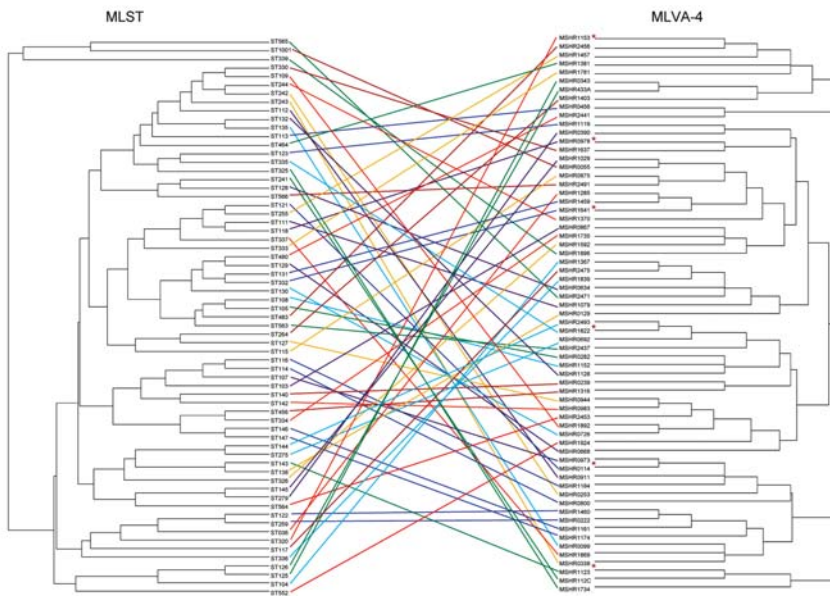
Figure 1. Comparison of multilocus sequence typing (MLST) and 4-locus multilocus variable number tandem repeat analysis (MLVA-4) dendrograms for 65 *Burkholderia pseudomallei* isolates. MLST sequence type (ST) is shown for each isolate, with the corresponding isolate number listed for the MLVA-4 profile and shown by the colored lines. The red asterisks indicate 6 isolates that represent diversity of MLVA-4; these isolates were used to calibrate the dendrogram in Figure 3.

contamination of a container of detergent (*14*). However, such outbreaks are rare, and we have shown that, in the melioidosis-endemic region of northern Australia, case clusters during extreme weather events are usually not genetically linked by PFGE (*8*). These clusters simply reflect the close association between rainfall and infection from the diverse range of *B. pseudomallei* strains in soil and surface water.

Recently, MLST has enabled new insights into regional and global epidemiology of melioidosis (*7,16,18–20*). Although there is excellent congruence between PFGE and MLST, with PFGE and MLST providing similar results for local epidemiologic investigations (*16*), MLST has the major advantage of absolute comparative ability across

laboratories through the MLST website and unambiguous sequence type characterization.

Ribotyping and PFGE take several days to generate results, and MLST is expensive and requires sequencing and analysis capability. PCR-based typing methods have enabled more rapid availability of results. Randomly amplified polymorphic DNA (RAPD) analysis has been used to analyze relationships between clinical and environmental *B. pseudomallei* (*21,22*). However, it is not possible to make valid comparisons of RAPD results between laboratories and sometimes even between runs in the same laboratory. Thus, despite the speed of RAPD, we no longer use it.

Analyzing bacterial genomes for VNTRs has enabled MLVA assays to be developed to differentiate among me-
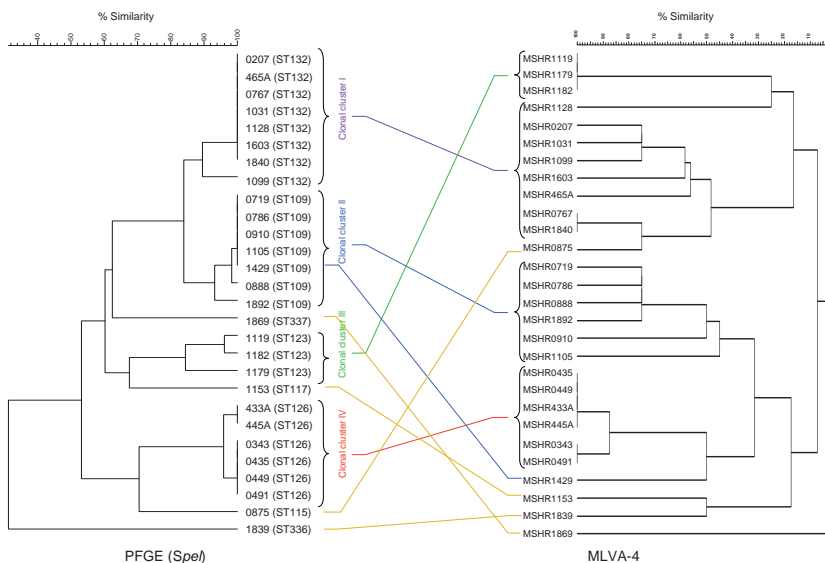
Figure 2. Comparison of pulsed-field gel electrophoresis (PFGE) and 4-locus multilocus variable number tandem repeat analysis (MLVA-4) profiles for isolates in 4 clonal groups (see text for details). Isolate number with its MLST sequence type (ST) is listed for each isolate on the PFGE profile, with the corresponding isolate number listed for the MLVA-4 profile. Four unrelated isolates are included for comparison: 0875 (ST115), 1869 (ST337), 1839 (ST336) and 1153 (ST117).
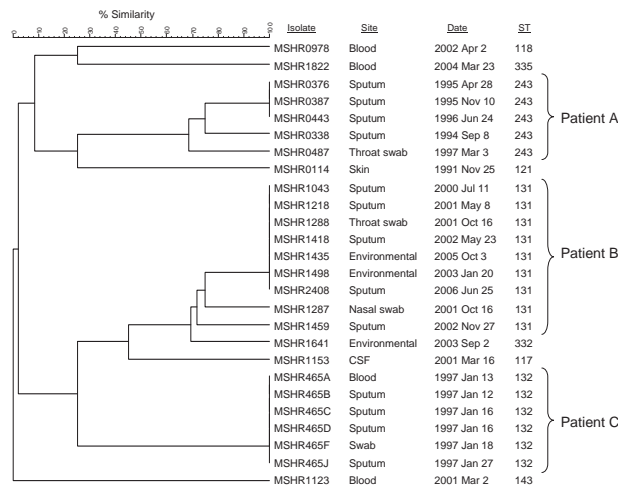
Figure 3. Dendrogram showing 4-locus multilocus variable number tandem repeat analysis profiles for isolates from 3 patients with melioidosis, with isolate number and multilocus sequence typing sequence type (ST) listed (see text for details). Six isolates used to calibrate the dendrogram are indicated by asterisks in Figure 1 and listed in Table 2. CSF, cerebrospinal fluid.

thicillin-resistant *Staphylococcus aureus* strains that are indistinguishable by PFGE (*23*) and to differentiate *Neisseria meningitidis* strains with identical MLST STs (*24*). Liu et al. developed the first MLVA system for *B. pseudomallei* (*25*). They selected 5 VNTR loci from the *B. pseudomallei* genome to include in a multiplex PCR–based MLVA that enabled them to demonstrate extensive diversity among 32 *B. pseudomallei* strains obtained during an unprecedented 4-month increase in melioidosis cases in Singapore in early 2004. Their results clearly excluded a point-source outbreak and suggested that the case cluster was related to the particularly high rainfall that occurred that year.

B. pseudomallei contains numerous VNTRs. Using a 32 VNTR system, U'Ren et al. showed extensive diversity within a global *B. pseudomallei* isolate set (*26*). When 30 of these VNTR loci were used to analyze 9 epidemiologically related *B. pseudomallei* isolate sets, fine-scale diversity was found even among closely related strains, including sequential isolates from persons (*13*). We sought to develop a rapid and robust minimum loci *B. pseudomallei* MLVA that differentiated unrelated strains and maintained the ability to link isolates from a point-source outbreak. Our approach was similar to that developed for MLVA of *N. meningitides*, in which an 8-locus system was used to look at the global epidemiology, with clustering similar to that obtained with MLST. In this system, 4 highly variable VNTR loci were then chosen to analyze *N. meningitides* serogroup C strains collected during a meningococcal outbreak in the Netherlands (*24*).

Our 4-locus MLVA for *B. pseudomallei* separated all 65 MLST STs analyzed. In addition to being highly dis-

criminatory, the MLVA-4 had good specificity in clustering genetically linked *B. pseudomallei* strains and performed as well as PFGE in identifying clonal clusters. In particular, MVLA-4 could distinguish between epidemiologically unlinked strains that were identical by MLST and PFGE (groups I and II; Figure 2), while isolates from confirmed point-source outbreaks (groups III and IV; Figure 2) were either identical or closely clustered. Similarly, multiple isolates from a patient with acute disease obtained over 2 weeks were all identical (patient C; Figure 3), and those recovered over a much longer period from patients with chronic disease were closely clustered but showed some diversification (patients A and B, chronic disease over years; Figure 3).

Because PFGE takes ≥5 days to obtain results, alternative typing methods are required to rapidly determine whether a cluster of melioidosis cases is genetically linked and therefore potentially an outbreak that requires an urgent public health response. We recently demonstrated that BOX-PCR can perform similarly to PFGE and MLST in typing *B. pseudomallei*, with the ability to usually discriminate between unrelated isolates, while also showing relatedness of epidemiologically linked isolates (*11*). However, although BOX-PCR can provide results within 10 hours of a laboratory receiving the bacterial strains, it is less reproducible than PFGE, and a reliable comparison of BOX-PCR results between laboratories is not possible (*27*). We found variation in BOX-PCR results when we compared results from different PCR machines in our own laboratory and band-density differentials dependent on DNA template concentration (*11*).

MLVA-4 results are generally reproducible and can be obtained quickly (*24*). In the initial *B. pseudomallei* MLVA used to investigate the Singapore cluster, agarose gel electrophoresis was used to size multiplexed PCR products and enabled analysis on the basis of the VNTR banding profile (*25*). However, use of a DNA sequencer for simultaneous sizing of the 4 fluorescently labeled PCR products enables ≥16 isolates to be analyzed in 1 run with our MLVA-4, and results are potentially available 8 hours after receipt of bacterial strains. For related but not identical MLVA-4 patterns, we assessed the specificity of strain clustering by generating dendrograms that compared strains in question with 6 reference strains that represented the considerable diversity seen on MLVA-4 (Figures 1, 3). Table 2 provides fragment size and repeat copy number (MLVA-4 code) data on these 6 strains for use as standards by other laboratories in generating their own MLVA-4 results for their own *B. pseudomallei* strains, with potential for direct comparison of MLVA-4 results between different laboratories. Subsequently, MLST can be used to verify relatedness of strains through the MLST database.

In summary, we have developed a simplified 4-locus MLVA that compares favorably with PFGE and MLST.

This analysis can be used to recognize or exclude a point-source outbreak of melioidosis within 8 hours of receipt of *B. pseudomallei* strains.

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Simian T-Lymphotropic Virus Diversity among Nonhuman Primates, Cameroon

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Cross-species transmission of retroviruses is common in Cameroon. To determine risk for simian T-cell lymphotropic virus (STLV) transmission from nonhuman primates to hunters, we examined 170 hunter-collected dried blood spots (DBS) from 12 species for STLV. PCR with generic *tax* and group-specific long terminal repeat primers showed that 12 (7%) specimens from 4 nonhuman primate species were infected with STLV. Phylogenetic analyses showed broad diversity of STLV, including novel STLV-1 and STLV-3 sequences and a highly divergent STLV-3 subtype found in *Cercopithecus mona* and *C. nictitans* monkeys. Screening of peripheral blood mononuclear cell DNA from 63 HTLV-seroreactive, PCR-negative hunters did not identify human infections with this divergent STLV-3. Therefore, hunter-collected DBS can effectively capture STLV diversity at the point where pathogen spillover occurs. Broad screening using this relatively easy collection strategy has potential for large-scale monitoring of retrovirus cross-species transmission among highly exposed human populations.

Primate T-lymphotropic viruses (PTLVs) are composed of simian and human T-lymphotropic viruses (STLVs and HTLVs, respectively). To date, only 4 major PTLV groups have been identified. PTLV-1, PTLV-2, and

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PTLV-3 include human (HTLV-1, HTLV-2, and HTLV-3) and simian (STLV-1, STLV-2, and STLV-3) viruses (*1–6*). PTLV-4 consists of only HTLV-4, which was recently reported in a person in Cameroon known to have been exposed to nonhuman primates (NHPs) (*7*). A simian counterpart of this virus has yet to be identified. More recently, a highly divergent STLV-1–like virus from captive macaques (*Macaca arctoides*) has been described (*8*); further analysis suggests a possible new lineage outside the diversity of PTLV-1, provisionally named STLV-5 (*9*).

Both HTLV-1 and HTLV-2 have spread globally and are pathogenic in humans (*10–13*). HTLV-1 causes adult T-cell leukemia/lymphoma, HTLV-1–associated myelopathy/tropical spastic paraparesis, and other inflammatory diseases in <5% of those infected (*2,11,13*). HTLV-2 is less pathogenic than HTLV-1 but has been associated with a neurologic disease similar to HTLV-1–associated myelopathy/tropical spastic paraparesis (*10,12*). HTLV-1 and HTLV-2 are known to be transmitted by sexual contact, breast-feeding, and exposure to contaminated blood or blood products through transfusion and injection drug use (*11–13*). Less is known about the transmissibility and pathogenicity of HTLV-3 and HTLV-4. Nevertheless, recent full-length sequence analysis of the HTLV-3 (*14,15*) and HTLV-4 genomes (W.M. Switzer et al., unpub. data) suggested ancient origins of these viruses and showed functional motifs that affect viral expression and possibly oncogenesis (*14,15*; W.M. Switzer et al., unpub. data).

The recent discovery of HTLV-3 and HTLV-4 demonstrates that the diversity of PTLV is far from understood

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(7). Studies have shown that the diversity of HTLV is directly related to the genetic diversity of the STLVs from which the primary zoonotic infection originated (5,16). Every HTLV-1 subtype except A is composed of genetically related HTLV-1 and STLV-1 strains from many different primate species, all found geographically near each other. Similarly, PTLV-3s exhibit broad diversity among NHPs in the wild; currently, 3 subtypes have been suggested according to the geographic origin of the strains (17): East African STLV-3 subtype A includes STLV-3 (PH969) found in a baboon (*Papio hamadryas*) from Eritrea (18) and from captive gelada baboons (*Theropithecus gelada*) (19); West and Central African STLV-3 subtype B includes STLV-3 (CTO-604) and STLV-3 (CTO-602) found among mangabeys (*Cercocebus torquatus*) from Cameroon (20) and STLV-3 (PPAF3) from baboons (*P. hamadryas papio*) from Senegal (17); and Central African STLV-3 subtype C includes divergent strains (Cni217 and Cni227) from *Cercopithecus nictitans* monkeys from Cameroon (21). Together, this clustering by geography rather than host species suggests the ease with which STLVs are transmitted among NHPs and possibly to humans (2,3,5,22,23).

We used a hunter-based field surveillance approach to investigate STLV diversity among primate bushmeat samples collected from 12 NHP species in different locations in Cameroon. We also sampled NHPs in the surrounding region for the STLV source of the HTLV-4–infected individual. In addition, we examined the utility of using dried blood spots (DBS) in the field for surveillance of cross-species transmission of retroviruses.

Materials and Methods

Sample Collection and Preparation

Before the study began, Institutional Animal Care and Use Committee approvals were obtained. Self-identified hunters from 4 study sites in southern Cameroon volunteered to collect DBS from freshly hunted NHP bushmeat (Figure 1). Hunters were educated about the risks associated with direct contact with NHPs and about appropriate prevention measures. Preliminary identification of hunted species was undertaken by using pictographs of NHPs common in the region (24). Confirmation of species was performed by analysis of mitochondrial cytochrome oxidase subunit II and/or glucose-6-phosphate dehydrogenase sequences (25,26). Over 2 years, a total of 362 DBS from hunted NHPs were collected on Whatman filter paper (Kent, UK), air dried, and stored locally at room temperature in envelopes with dessicant until processed. Nucleic acids were extracted by using the NucliSens nucleic acid isolation kits (bioMérieux, Durham, NC, USA). DNA quality and yield were determined by semiquantitative PCR amplification of the β-actin gene as previously described (27,28). DNA
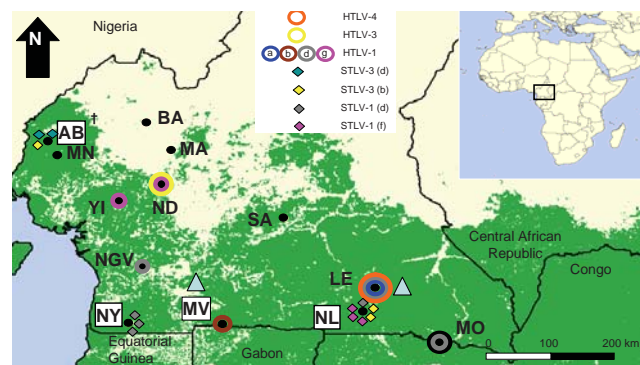
Figure 1. Distribution of primate T-lymphotropic viruses identified in humans and nonhuman primates from rural villages and forests in southern Cameroon. Colored circles and diamonds correspond to human (HTLVs) and simian T-lymphotropic viruses (STLVs) (subtypes), respectively, found at each study site in the current study and reported previously (7). Shaded triangles indicate approximate sampling sites where STLV-3–like strains have been reported by others (9). The 4 locations where Old World monkey and prosimian species were sampled in this study are boxed in white. AB, Abat; MV, Mvangan; NY, Nyabissan; NL, Ngoila; MN, Manyemen; BA, Bangourain; MA, Massangam; YI, Yingui; ND, Ndikinimeki; NGV, Ngovayan; SA, Sobia; LE, Lomie; MO, Mouloundou.

preparation and PCR assays were performed in different laboratories specifically outfitted for processing and testing of NHP samples only, according to established precautions to prevent contamination. Specimens were coded by using a strategy previously described (15).

PTLV Sequence Detection and Sequence Analysis

DNA samples from NHPs were tested for *tax* sequences by using generic and nested PCR assays capable of detecting viruses from all 4 major PTLV groups (7,19,27). Phylogenetic resolution was achieved by analysis of long terminal repeat (LTR) sequences using PTLV group-specific primers (7). PCR amplification of overlapping regions of the 5′ and 3′ STLV-1 LTR (4) and partial STLV-3 LTR (7,19) sequences were performed using primers and conditions reported elsewhere. A PCR-based genome-walking approach (15) was used to obtain partial viral genome fragments of a highly divergent PTLV from monkeys Cmo8699AB and Cni7867AB (Table 1). (NHPs are coded as follows: the first letter of the genus is followed by the first 2 letters of the species name, e.g., Cag, *Cercocebus agilis;* Cni, *C. nictitans;* Cmo, *C. mona;* and Lal, *Lophocebus albigena.* The last 2 letters in the code indicate the study site, e.g., AB, Abat; MV, Mvangan.)

To screen humans for the divergent STLV-3 subtype, we developed a nested PCR assay based on STLV-3 (Cmo8699AB) *tax* sequences. Similar strategies have been used to screen for the novel HTLV-3 and HTLV-4 viruses in NHP hunters from Cameroon (1,7). DNA for PCR test-

Table 1. Nucleotide sequences of primer sets used to amplify *tax* and long terminal repeat sequences of simian T-lymphotropic virus–3 (Cmo8699AB and Cni7867AB)*

Region	Primer set	Forward primer and sequence (5′ → 3′)	Reverse primer and sequence (5′ → 3′)	bp
tax	Outer	8699TF1 GTACCCTGTCTACGTTTTCGGCGAT	PGTAXR1 GAIGAYTGIASTACYAAAGATGGCTG	779
	Inner	8699TF2 TTACTGGCCACCTGTCCTGAACAC	PGTAXR2 TTIGGGYAIGGICCGGAAATCAT	658
	Outer	P5TAXF3† CCCTCAAGGTCCTCACCCCGCCGC	P5TAXR3 TAACGGCCAGGTCATTGGAGGTGT	244
	Inner	P5TAXF2† AAGTTCCTCCCTCCTTCTTCCATG	P5TAXR1 TGGTAGAGGTATAAGCACACGATGGTG	174
tax-LTR	Outer	8699TF6 CATCCGGACCAACTAGGGGCCTTC	PGTATA1+2R1 TCCTGAACYGTCYYYRCGCTTTTATAG	721
	Inner	8699TF7‡ AACAAAAATCCCTACCAAACGCTT	PGTATA1+2R1 TCCTGAACYGTCYYYRCGCTTTTATAG	695
	Inner	8699TF8§ CAGCCCACCCGCGCACCAGTAATT	PGTATA1+2R1 TCCTGAACYGTCYYYRCGCTTTTATAG	589
LTR	Outer	8699LF3 CTCTGACGTCTCTCCCTGCCTTGT	PGPBSR1n ATCCCGGACGAGCCCCCA	612
	Inner	8699LF4 CCGGAAAAAACCTTAAACCACCCA	PGPBSR1n ATCCCGGACGAGCCCCCA	585

*bp, basepair; LTR, long terminal repeat; I, inosine; S, G/C; Y, T/C; R, A/G. Nonhuman primates are coded as follows: the first letter of the genus is followed by the first 2 letters of the species name: Cmo, *Cercopithecus mona*; Cni, *C. nictitans*. The last 2 letters in the code indicate the study site: AB, Abat.
†Primers used to screen human peripheral blood mononuclear cell DNA for simian T-lymphotropic virus-3 (Cmo8699AB)–like *tax* sequences.
‡Primer set used for Cni7867AB.
§Primer set used for Cmo8699AB.

ing was available from a previous study in which plasma from 63 hunters showed a range of seroreactivity to HTLV antigens by Western blot (WB; Genelabs Diagnostics 2.4 kit [*7*]). WB profiles were HTLV-1–like (n = 2), HTLV-2–like (n = 4), HTLV-positive but untypeable (n = 8), and HTLV-indeterminate (n = 49) (*7*). New STLV-3 (Cmo8699AB)-*tax* specific primers were designed to screen peripheral blood mononuclear cell DNA from all 63 hunters previously negative for sequences by using generic primers that can detect PTLV-1, PTLV-2, PTLV-3, and PTLV-4 (*7*).The assay could reliably detect 10 copies of STLV-3 (Cmo8699AB) *tax* plasmid sequences in a background of human DNA. STLV-3 (Cmo8699AB) *tax* sequences were not amplified from PTLV-1, PTLV-2, PTLV-3, and HTLV-4 cell line DNA or *tax*-containing plasmid DNA or from HTLV non-reactive blood donor DNA samples (data not shown), which shows the high sensitivity and specificity of the assay.

PCR products were purified by using QIAquick PCR or gel purification kits (QIAGEN, Valencia, CA, USA) and were either directly sequenced in both directions on an ABI 3130*xl* sequencer (Applied Biosystems, Foster City, CA, USA) or were sequenced after cloning into a TOPO vector (Invitrogen, Carlsbad, CA, USA). Sequence and phylogenetic analyses were performed according to methods previously described (*15*). Molecular dating of STLV-3 (Cmo8699AB) was based on an alignment of 881-bp *tax* sequences and used previously reported methods (*15*). GenBank accession numbers for the STLV-1 LTR, STLV-3 LTR, STLV-3 (Cmo8699AB) *tax*-LTR, and small *tax* sequences are EU152271–EU152276, EU152277–

EU152279, EU152280–EU152281, and EU152282–EU152293, respectively.

Results

A total of 362 DBS representing 12 NHP and prosimian species were collected (Figure 1), of which 215 (60%) were of adequate quality and quantity for nucleic acid extraction, and 170 (79%) of the 215 yielded adequate amplifiable DNA (Table 2). Blood clots and limited volumes of blood on some DBS accounted for poor DNA yield of some samples.

Because of the limited amount of DBS material available, we used a PCR assay that detects sequences from all 4 major PTLV groups. We observed a broad range of PTLV diversity over a wide geographic distribution. Of the 170 samples screened, 12 (7%) from 4 NHP species were positive for PTLV *tax* sequences (Table 3). Phylogenetic analysis of the short *tax* sequences from these 12 samples showed that 7 NHPs (2 *Cercocebus agilis* and 5 *C. nictitans* monkeys) were infected with STLV-1 and that 3 (*C. agilis*, *C. nictitans*, and *Lophocebus albigena* monkeys) were infected with STLV-3 (Figure 2; Table 3). We did not find any evidence of STLV-2, HTLV-4–like STLV, or dual STLV-1 and STLV-3 infections as have been found in *C. agilis* monkeys in other studies (*25*).

Samples Cmo8699AB and Cni7867AB, each collected near the same village but from 2 different NHP species, contained nearly identical STLV sequences with highest nucleotide identity to viruses in the PTLV-3 group, but they exhibited high divergence in this small region of *tax* (Figure 2;

Table 2. Primate T-lymphotropic virus distribution among wild simian and prosimian species, Cameroon*

Taxonomic name (common name)	DBS extracted, no.	β-actin positive, no. (%)	*tax* positive,† no. (%)	STLV-1 LTR positive, no.	STLV-3 LTR positive, no.
Old World monkeys					
Cercocebus agilis (agile mangabey)	6	3 (50)	3 (100)	2	1
C. cephus (moustached monkey)	41	32 (78)	0	0	0
C. mona (mona monkey)	40	36 (90)	1(2.7)	0	1
C. neglectus (de Brazza's monkey)	1	1 (100)	0	0	0
C. nictitans (spot-nosed monkey)	98	73 (74.5)	7 (9.6)	4	2
C. pogonias (crowned monkey)	9	8 (88.8)	0	0	0
Colobus guereza (guereza colobus)	3	2 (66.7)	0	0	0
Lophocebus albigena (gray-cheeked monkey)	10	9 (90)	1 (11.1)	0	1
Prosimian					
Arctocebus aureus (golden angwantibo)	2	1 (50)	0	0	0
A. calabarensis (calabar angwantibo)	2	2 (100)	0	0	0
Galago alleni (Allen's galago)	1	1 (100)	0	0	0
Perodicticus potto (potto)	2	2 (100)	0	0	0
Total	215	170 (79.1)	12 (7.1)	6 (3.5)	5 (2.9)

*DBS, dried blood spots; STLV, simian T-lymphotropic virus; LTR, long terminal repeat.
†Samples negative for ß-actin sequences were not tested for primate T-lymphotropic virus sequences.

Table 4). BLAST analysis (www.ncbi.nlm.nih.gov/blast/ Blast.cgi) of these divergent *tax* sequences identified sequence similarity (≈92%–93%) to short STLV-3–like *tax* sequences (≈219 bp) from 4 *C. nictitans* monkeys from southern Cameroon (Cni217, Cni227, Cni3034, and Cni3038; GenBank accession nos. AY039033, AF412120, AM746663, and AM746660, respectively) (Table 4) (*9,21*). However, further phylogenetic analysis of STLV-3 (Cmo8699AB) and STLV-3 (Cni7867AB), including the small *tax* sequences from 3 of the 4 *C. nictitans* monkeys (Cni3034 was omitted because it had a shorter but identical *tax* sequence to Cni3038) and from other STLV-3–infected species (*L. albigena*, *C. agilis*, and *C. cephus*) from the same region (*9,21*), showed that our viruses clustered tightly with high bootstrap support (99%) as a distinct monophyletic subtype of STLV-3 (Figure 3). Because nucleotide divergence is generally <3% within viral subtypes and up to 15% between viral subtypes in the *tax* region (*7*), the 7% divergence seen in the *tax* sequences of STLV-3 (Cmo8699AB)

and STLV-3 (Cni7867AB), along with the clustering of these viruses outside the diversity of other STLV-3–like viruses (*9,21*), suggested the identification of a new and highly divergent PTLV-3 subtype (Figure 3; Table 4).

Phylogenetic Resolution of a Novel PTLV-3 Subtype

The identification of highly divergent STLV-3–like sequences in Cmo8699AB and Cni7867AB was investigated further by additional analyses of a larger *tax* sequence (1,015 bp). Both *tax* sequences were nearly identical (99.9%) despite nucleic acid extraction, PCR amplification, and sequencing for both animals all being performed on different days. Analysis of mitochondrial DNA sequences also confirmed the *Cercopithecus* species of each monkey and the absence of admixtures of specimens from different NHP species. STLV-3 (Cmo8699AB) *tax* sequences share 72%–74% nucleotide identity with PTLV-1, PTLV-2, and PTLV-4, but they have the highest nucleotide identity to the PTLV-3 group (82%–84%) in this highly conserved

Table 3. Primate T-lymphotropic virus diversity and geographic distribution among wild nonhuman primates, Cameroon*

No.	Code	Species (common name)	Site	Province	PTLV (subtype)
1	Cag9812NL	*Cercopithecus agilis* (agile mangabey)	Ngoila	East	STLV-1 (f)
2	Cag9813NL	*C. agilis*	Ngoila	East	STLV-1 (f)
3	Cag9748NL	*C. agilis*	Ngoila	East	STLV-3 (b)
4	Cmo8699AB	*C. mona* (mona monkey)	Abat	Southwest	STLV-3 (d)
5	Cni10026NL	*C. nictitans* (spot-nosed monkey)	Ngoila	East	STLV-1†
6	Cni10225NL	*C. nictitans*	Ngoila	East	STLV-1 (d)
7	Cni8284NY	*C. nictitans*	Nyabissan	South	STLV-1 (d)
8	Cni8286NY	*C. nictitans*	Nyabissan	South	STLV-1 (d)
9	Cni8348NY	*C. nictitans*	Nyabissan	South	STLV-1 (d)
10	Cni7882AB	*C. nictitans*	Abat	Southwest	STLV-3 (b)
11	Cni7867AB	*C. nictitans*	Abat	Southwest	STLV-3 (d)
12	Lal9589NL	*Lophocebus albigena* (gray-cheeked monkey)	Ngoila	East	STLV-3 (b)

*PTLV, primate T-lymphotropic virus; STLV, simian T-lymphotropic virus. Nonhuman primates are coded as follows: the first letter of the genus is followed by the first 2 letters of the species name: Cag, *C. agilis*; Cmo, *C. mona*; Cni, *C. nictitans*.; Lal, *L. albigena*. The last 2 letters in the code indicate the study site: AB, Abat; NL, Ngoila; NY, Nyabissan.
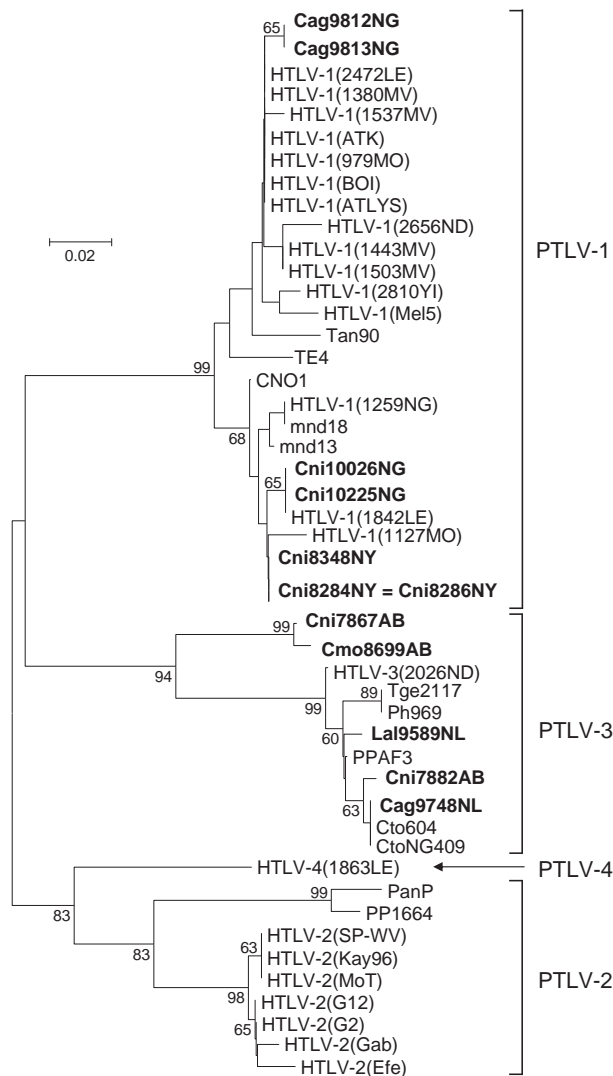†Subtype not determined.

Figure 2. Primate T-lymphotropic virus (PTLV) phylogeny inferred by using 161-bp *tax* sequences. New sequences from nonhuman primates (NHPs) from Cameroon in this study are in **boldface**. Support for the branching order was determined by 1,000 bootstrap replicates; only values ≥60% are shown. Branch lengths are proportional to the evolutionary distance (scale bar) between the taxa. Nonhuman primates are coded as follows: the first letter of the genus is followed by the first 2 letters of the species name: Cmo, *Cercopithecus mona;* Cni, *Cercopithecus nictitans;* Cto, *Cercocebus torquatus;* Ppa, *Papio papio;* Ph, *Papio hamadryas;* Tge, *Theropithecus gelada.* Cag, *Cercocebus agilis;* Lal, *Lophocebus albigena*; Mnd and msp, *Mandrillus sphinx;* PanP and PP, *Pan paniscus*; Ptr, *Pan troglodytes*; Ggo, *Gorilla gorilla*; Tan, tantalus monkey; Cag, *Cercocebus agilis*; Mar, *Macaca arctoides*; Pha, *Papio hamadryas*; Pan, *Papio anubis*; Bab, baboon; HYB, hybrid baboon (Pha X Pan); Cae, *Chlorocebus aethiops* (AGM, African green monkey); Cpo, *Cercopithecus pogonias*; Cmi, *Cercopithecus mitis*; Cce, *Cercopithecus cephus*; Ang, *Allenopithecus nigroviridis*; Wrc, Western red colobus. The last 2 letters in the code indicate the study site: AB, Abat; MV, Mvangan; NY, Nyabissan; NL, Ngoila; MN, Manyemen; BA, Bangourain; MA, Massangam; YI, Yingui; ND, Ndikinimeki; NG, Ngovayan; SA, Sobia; LE, Lomie; MO, Mouloundou.

region where intragroup sequence identity is typically >90%. Phylogenetic analysis of 881-bp *tax* sequences (Figure 4) from these 2 monkeys with other PTLVs, using bovine leukemia virus as an outgroup, inferred a new lineage with high bootstrap support (99%) from the diversity of other PTLV-3 subtypes (larger *tax* sequences representing PTLV-3 subtype C were not available for inclusion in this analysis), which suggests a long, independent evolution of this divergent virus.

Similar PTLV-3 tree topologies were obtained by analysis of 275-bp LTR sequences (Figure 5) in which STLV-3 (Cmo8699AB) and STLV-3 (Cni7867AB) had only 70%–74% identity to LTRs from other members of the PTLV-3 group that share >84% nucleotide identity between subtypes A and B (data not shown). LTR sequences from other STLV-3-infected *C. agilis* and *C. nictitans* monkeys from Cameroon reported elsewhere were not available from GenBank (*9,21,25*) and thus were not included in the current phylogenetic analysis. Combined, the phylogenetic analyses of the *tax* (Figures 3, 4) and LTR (Figure 5) sequences show that STLV-3 (Cmo8699AB) and STLV-3 (Cni7867AB) each form a distinct cluster with high bootstrap support from the other known PTLV-3 subtypes. On the basis of nomenclature proposed by others (*17*), our results suggest that these viruses are members of a novel PTLV-3 subtype that we tentatively name as STLV-3 West African subtype D.

Origin of STLV-3 (Cmo8699AB)

To estimate the divergence times of the most recent common ancestor of STLV-3 (Cmo8699AB), we performed additional molecular analyses. We found that the molecular clock hypothesis was not rejected for the 881-bp alignment of PTLV and bovine leukemia virus *tax* sequences in both PAUP* (http://paup.csit.fsu.edu) and Tree-Puzzle (www.tree-puzzle.de) analyses (p = 0.012 and 0.858, respectively), which is consistent with results obtained recently by others (*29*). Using a molecular clock model and a tree calibration date estimated for the origin of Melanesian HTLV-1 ≈40,000–60,000 years ago (*15,19,29,30*), we inferred the evolutionary rate for PTLV to be 9.17×10^{-7} to 1.38×10^{-6} substitutions/site/year, which is consistent with rates determined previously both with and without a molecular clock model of evolution (*15,17,20,29–31*). The evolutionary rate for STLV-3 (Cmo8699AB) is estimated to be 2.11×10^{-6} to 3.16×10^{-6}, and the most common recent ancestor is inferred to have occurred ≈92,072–138,560 years ago, which suggests an ancient origin and perhaps the identification of one of the oldest viruses in the PTLV-3 group.

Broad STLV-3 Diversity in Wild NHPs

Sequence analysis of the STLV-3 LTR sequences from Cni7882AB, Cag9748NL, and Lal9589NL showed that all

Table 4. High genetic diversity of novel STLV-3 (subtype D) *tax* sequences compared to prototypical PTLV-3s*

Nonhuman primate	Subtype D		Subtype C				Subtype B				Subtype A	
	Cmo 8699AB†	Cni 7867AB†	Cni 217‡	Cni 227‡	Cni 3034§	Cni 3038¶	2026 ND	Cto604	CtoNG 409	PPAF3	Ph969	Tge 2117
Cmo8699AB	–	99.9	**92.7**	**93.2**	**93.5**	**93.1**	**82.7**	**83.4**	**83.5**	**83.5**	**84.5**	**84.2**
Cni7867AB		–	**92.7**	**93.2**	**93.5**	**93.1**	**82.7**	**83.4**	**83.5**	**83.5**	**84.5**	**84.2**
Cni217			–	99.5	98.2	98.5	**84.5**	**86.3**	**88.1**	**86.8**	**88.6**	**88.1**
Cni227				–	98.8	99.1	**84.9**	**86.8**	**87.7**	**87.2**	**89.0**	**88.6**
Cni3034					–	100.0	**82.2**	**82.4**	**82.8**	**83.6**	**83.9**	**83.7**
Cni3038						–	**82.5**	**82.7**	**83.1**	**83.7**	**84.1**	**83.9**
2026ND							–	91.6	93.0	94.1	**87.0**	**90.4**
Cto604								–	92.4	92.5	**87.5**	**92.0**
CtoNG409									–	94.2	**86.8**	**90.7**
PPAF3										–	**88.5**	**90.8**
Ph969											–	95.8
Tge2117												–

*STLVs, simian T-lymphotropic viruses; PTLVs, primate T-lymphotropic viruses. **Boldface** indicates intersubgroup identities; shading indicates intrasubgroup identities. Nonhuman primates are coded as follows: the first letter of the genus is followed by the first 2 letters of the species name: Cmo, *Cercopithecus mona;* Cni, *C. nictitans;* Cto, *Cerococebus torquatus*; Ppa, *Papio papio*; Ph, *Papio hamadryas*; Tge, *Theropithecus gelada*. The last 2 letters in the code indicate the study site: AB, Abat; ND, Ndikinimeki.
†Partial *tax* sequence (1015 bp).
‡Partial *tax* sequence (219 bp).
§Partial *tax* sequence (170 bp).
¶Partial *tax* sequence (202 bp).

Figure 3. Identification of a novel primate T-lymphotropic virus (PTLV)–3 subtype by phylogenetic inference of 202-bp *tax* sequences with PTLV prototypes and partial sequences from 3 *Cercopithecus nictitans* (Cni217, Cni227, and Cni3038) reported elsewhere (*9,21*) and those identified in the current study (in **boldface**). GenBank accession numbers for the previously reported partial simian T-lymphotropic virus (STLV)–3 *tax* sequences included in this analysis are AY039033, AF412120, and AM746647–AM746673. Support for the branching order was determined by 1,000 bootstrap replicates; only values ≥60% are shown. Branch lengths are proportional to the evolutionary distance (scale bar) between the taxa. See Figure 2 legend for abbreviations.

were infected with distinct STLV-3s. LTR sequences (283 bp) from animal Cag9748NL shared the greatest identity (≥97%) with those from HTLV-3 (Pyl43) and STLV-3 (Cto604) from a red-capped mangabey from Cameroon (*1,20*). The 282-bp LTR sequence from Cni7882AB shared the highest nucleotide identity (99%) to STLV-3 (CtoNG409), a red-capped mangabey from neighboring Nigeria (*31*). The phylogeographic clustering of these sequences supports further the proposed subtype classification of STLV-3 by geographic origin rather than by host species (*17,19,20,25,31*). In contrast, the 432-bp LTR sequence from *L. albigena* mangabeys (Lal9589NL) was more divergent; it shared only 10%–16% nucleotide identity with all PTLV-3 LTR sequences. Similar to the phylogenetic relationships inferred with the small *tax* sequences, the LTR sequence from *L. albigena* mangabeys (Lal9589NL) formed a new lineage within the diversity of other PTLV-3 sequences from west-central Africa (Figure 5). Although these results need to be confirmed with additional LTR sequences from this virus and from other STLV-3–infected *L. albigena* mangabeys (*9*), our findings demonstrate a host range and geographic distribution of STLV-3 that is more widespread than previously considered.

Phylogenetic Analysis of STLV-1 Diversity

To investigate further the genetic relationships inferred with the small PTLV-1–like *tax* sequences, we obtained LTR sequences for 6 of 7 PTLV-1–positive samples by using established primer-pair combinations (*3,4,7*). Phylogenetic analysis of these sequences, including those identified from our study of infected NHP hunters in Cameroon (*7*), showed that 4 sequences from *C. nictitans* mon-
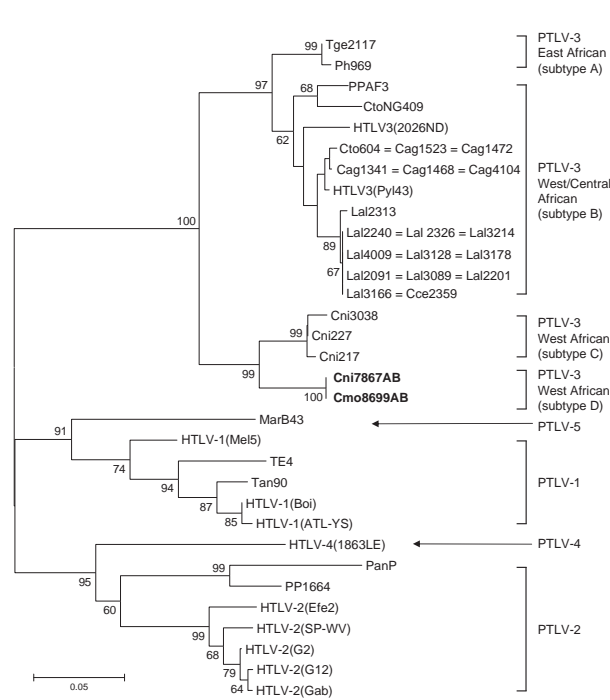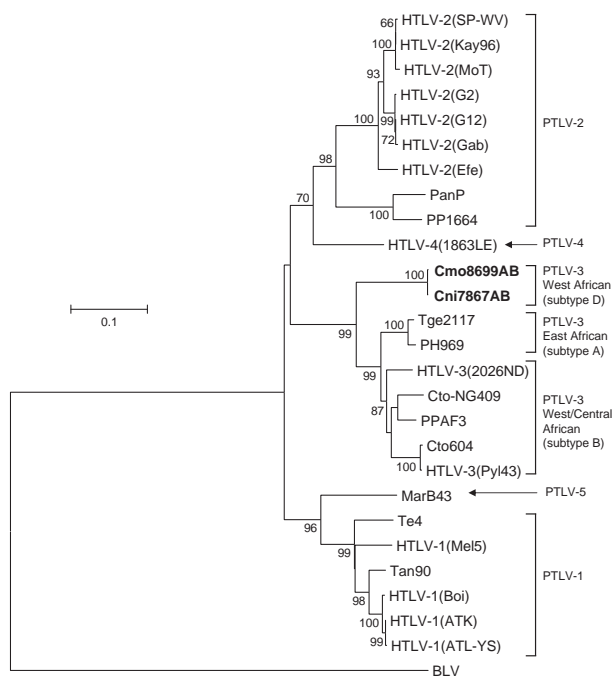
Figure 4. Identification of a novel primate T-lymphotropic virus (PTLV) subtype by phylogenetic inference of 881-bp *tax* sequences from prototypical PTLVs. Bovine leukemia virus (BLV) *tax* sequences were used as an outgroup in the maximum-likelihood analysis. New sequences from this study are in **boldface**. Support for the branching order was determined by 1,000 bootstrap replicates; only values ≥60% are shown. Branch lengths are proportional to the evolutionary distance (scale bar) between the taxa. See Figure 2 legend for abbreviations.

Absence of Novel STLV-3 Subtype Sequences in NHP Hunters

Given the prevalence of the STLV-3 subtype D virus in at least 2 monkey species in Cameroon, we investigated whether this new subtype was also present among NHP hunters in Cameroon. Peripheral blood mononuclear cell DNA samples were available from a previous study of 63 NHP hunters who had a wide range of WB seroreactivity to HTLV (*7*). HTLV sequences were not previously detected in the DNA of these persons when either generic or group-specific primers were used (*7*). All 63 NHP hunters were also negative for STLV-3 (Cmo8699AB) *tax*-specific sequences, which suggests the absence of this virus in this subset of persons with broad WB seroreactivity to HTLV.

Discussion

Widespread exposure to a broad range of NHP body fluids and tissues encountered during hunting, butchering, or keeping primates as pets has been implicated in the emergence of 3 different retrovirus genera: HIV, HTLV, and, more recently, simian foamy virus (*2–5,7,16,28,32*). Although little is known about the public health implications of simian foamy virus infection, the social, medical, political, and economic consequences of HIV and HTLV global spread and pathogenicity after cross-species transmission are enormous. The recent discovery of HTLV-3 and HTLV-4 in NHP hunters from Cameroon doubles the number of known deltaretroviruses in humans (*7*). This

keys all clustered in the central African HTLV-1 subtype D clade, consisting of STLV-1 from *Mandrillus sphinx* and *Cercopithecus pogonias* monkeys and HTLV-1 sequences from Cameroon (Figure 6). The STLV-1 (Cni10225NL) LTR sequence was phylogenetically closest to the HTLV-1 (1842LE) strain from an NHP hunter from Cameroon (*7*) (Figure 6). Similarly, LTR sequences from 2 *C. agilis* (Cag9812NL and Cag9813NL) monkeys clustered within the HTLV-1F clade (Figure 6). Combined, these results support further the primate origin of the HTLV-1D and -1F subtypes. We were unable to amplify STLV-1 LTR sequences from DBS samples from a *C. nictitans* monkey (Cni10026NL) that was positive for STLV-1 *tax* sequences, possibly because of low viral load in this animal, lower sensitivity of the LTR primers, or genetic variances at the LTR primer binding sites. The absence of STLV-1 LTR sequences in this monkey is not likely to have resulted from infection with an STLV-1/STLV-3 recombinant after dual infection of animal Cni10026NL with both viruses because samples from this animal were repeatedly negative for STLV-3 *tax* and LTR sequences.

Figure 5. Identification of a novel primate T-lymphotropic virus (PTLV)–3 subtype by phylogenetic analysis of 275-bp long terminal repeat (LTR) sequences. LTR sequences for PTLV-3 subtype C were not available for this analysis. New sequences from this study are in **boldface**. Support for the branching order was determined by 1,000 bootstrap replicates; only values ≥60% are shown. Branch lengths are proportional to the evolutionary distance (scale bar) between the taxa. BLV, bovine leukemia virus. See Figure 2 legend for additional abbreviations.

Figure 6. Inferred phylogenetic relationships of primate T-lymphotropic virus (PTLV)–1 long terminal repeat (LTR) sequences by neighbor-joining analysis. Sequences from wild nonhuman primates (NHPs) in Cameroon generated in the current study are in **boldface**. Human T-lymphotropic virus–1 seqences are italicized. Support for the branching order was determined by 1,000 bootstrap replicates; only values ≥60% are shown. STLV, simian T-lymphotropic virus. See Figure 2 legend for additional abbreviations.

same study also identified novel STLV-1–like infections in NHP hunters (7). These discoveries demonstrate that the diversity of PTLV is far from understood and that zoonotic infection with STLV continues in persons exposed to NHPs (7). Thus, understanding the diversity, prevalence, and geographic range of STLV infection in areas where frequent contact with wild NHPs is common provides useful information about the origin and emergence of HTLV and the risks for exposure to these and possibly other simian viruses.

We demonstrated that monkeys from 3 distant locations in the rain forests of southern Cameroon are infected with a broad range of highly diverse STLV. Our detection of a 7% prevalence of STLV infection among hunted wild monkeys is comparable to the 8%–11% seroreactivity to PTLV recently found in monkey and ape samples collected mostly at urban bushmeat markets in Cameroon (9,25). Through analysis of LTR and larger *tax* sequences from *C. mona* and *C. nictitans* monkeys in our study, we have

identified new divergent STLV-3–like strains that form a unique PTLV-3 clade that we designated subtype D. Altogether, these results extend further the range of PTLV diversity and suggest a founder effect for PTLV evolutionary radiation in this region where most PTLV groups have been identified.

Given the propensity of STLV to cross species boundaries, the increased frequency of hunting and demand for primate bushmeat in Africa, and the apparent broad diversity of STLV subtypes in Cameroon (9,21), it is tempting to speculate that human infection with this unique STLV-3 subtype will or may have already occurred. However, PCR testing of DNA samples from Cameroon NHP hunters with broad HTLV WB patterns showed no evidence of STLV-3 (Cmo8699AB)–like infections. Possible explanations for this negative finding include the testing of only a limited number of available samples, an unknown sensitivity for serologic detection of this virus with assays used in our study (7), an unknown prevalence and host range of this virus in NHPs, and other factors such as low transmissibility to humans. Nevertheless, the discovery of this novel PTLV-3 subtype in 2 monkey species and an apparent ancient origin of this lineage suggest a possible wider distribution of this variant. Therefore, the ease with which STLVs can cross species barriers and potentially be transmitted during NHP-hunting practices warrants increased surveillance for human infection with this divergent subtype. A similar strategy involving intensified screening of NHP hunters was successful in the discovery of HTLV-3 (1,7) and HTLV-4 (7).

Finding a broad range of STLVs in simian DBS indicates that persons exposed to NHPs from Cameroon are at increased risk for infection with highly diverse STLV. Indeed, phylogenetic analysis of PTLV-1 LTR sequences shows that the new STLV-1 from *C. nictitans* monkeys identified in the current study is most similar to HTLV-1 from Cameroon NHP hunters (7). Similarly, the clustering of STLV-1 from *C. agilis* monkeys with LTR sequences obtained from a person from Liberia provides additional support for the primate origin of the HTLV-1F clade (33). Combined, these findings further support the hypothesis of active cross-species transmission of STLV to humans in this region (7).

Moreover, we show that use of DBS collected in the field in collaboration with hunters provides a good tool for surveillance of emerging retroviral infections at the NHP-hunter interface. Convenient and cost-effective, this collection strategy provides a unique opportunity to examine zoonotic transmission at the point where pathogen spillover occurs. In conjunction with longitudinal sampling of hunters, these collections have the potential to enable simultaneous documentation of both sides of a cross-species transmission event.

In summary, we found broad diversity of STLV in NHPs from Cameroon and identified a novel STLV-3 subtype. These results provide increasing evidence that the diversity and geographic distribution of PTLVs are much greater than previously thought. Bushmeat hunting, an ancient and common practice in many parts of Africa, is an ideal interface for cross-species transmission of retroviruses between NHPs and humans. Contact with body fluids and blood during hunting and butchering of NHP bushmeat exposes humans to a plethora of simian retroviruses, as demonstrated here and elsewhere (*7,23,25,32,34,35*), and increases the likelihood of emerging diseases in humans. To predict and possibly prevent the next retrovirus pandemic, expanded surveillance is needed for these and other retroviruses in their natural host reservoirs and in persons exposed to NHPs (*7,36,37*).

Mr Sintasath is a doctoral candidate at the Johns Hopkins Bloomberg School of Public Health. His primary research interests include zoonotic infections and the mechanisms of human disease emergence.

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Imported Malaria in Children in Industrialized Countries, 1992–2002

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Children account for an appreciable proportion of total imported malaria cases, yet few studies have quantified these cases, identified trends, or suggested evidence-based prevention strategies for this group of travelers. We therefore sought to identify numbers of cases and deaths, *Plasmodium* species, place of malaria acquisition, preventive measures used, and national origin of malaria in children. We analyzed retrospective data from Australia, Denmark, France, Germany, Italy, Japan, the Netherlands, Sweden, Switzerland, the United Kingdom, and the United States and data provided by the United Nations World Tourism Organization. During 1992–2002, >17,000 cases of imported malaria in children were reported in 11 countries where malaria is not endemic; most (>70%) had been acquired in Africa. Returning to country of origin to visit friends and relatives was a risk factor. Malaria prevention for children should be a responsibility of healthcare providers and should be subsidized for low-income travelers to high-risk areas.

M alaria is associated with high healthcare costs (*1*). Although several industrialized countries (e.g., most European countries, the United States, Australia, Japan) (*2*)

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are classified as malaria nonendemic, human migration and tourist travel to malaria-endemic regions are resulting in the importation of malaria (an estimated 30,000 cases/year) into these malaria-free countries (*3*). In the World Health Organization European Region, the number of imported cases rose from 1,500 in 1972 to 13,000 in 1999 (*4*). Children account for a considerable proportion of total malaria cases imported into the United States and Europe (*5*). International migration increased from 75 million in 1960 to 175 million in 2000 (*6*). Furthermore, the World Population Prospects report predicts a sharp increase in the number of persons who will migrate from southern (malaria-endemic) areas to northern (malaria-free) industrialized areas worldwide (*7*). This immigration trend also predetermines subsequent immigrant travel patterns. Immigrants returning to visit families in their home countries are at high risk for travel-related illness (*8–12*).

Malaria cases in children especially are increasing as more children travel and as the profile of immigrants changes (*13–16*). Our main study objectives were to evaluate the epidemiology of imported malaria in children in industrialized countries, identify trends and risk groups, and rank destinations according to malaria risk for children.

Methods

We collected retrospective data on imported malaria cases in children for the 11-year period January 1992 through December 2002. We requested data from 11 industrialized countries in which malaria is not endemic: Australia, Denmark, France, Germany, Italy, Japan, the Netherlands, Sweden, Switzerland, the United Kingdom, and the United States.

Malaria Cases

We defined imported malaria in a child as parasitologically confirmed malaria that had been acquired in a disease-

endemic area by a person ≤18 years of age and that was diagnosed after clinical disease had developed and when the person was in an industrialized country where the disease was not endemic. Data were collated directly from the countries' health authorities and consisted of aggregated case numbers of imported malaria in children by year (1992–2002), age group, sex, *Plasmodium* species, place of infection acquisition, number of deaths, national origin of patients, and preventive measures used during travel (i.e., chemoprophylaxis, bed nets, protective clothing, repellents).

We examined the distribution of malaria cases in children according to the variables of interest. Total cases were stratified according to *Plasmodium* species. On the basis of numbers of deaths and of *Plasmodium falciparum* cases, we calculated case-fatality ratio and 95% confidence intervals (CIs).

Traveler Statistics

The United Nations World Tourism Organization provided data on total numbers of travelers and extrapolated numbers of children ≤18 years of age who had traveled from >1 of the 11 industrialized countries in this study to malaria-endemic areas in Africa. Because exact numbers were not readily available, we assumed and used as proxy data the proportion of young travelers from the overall number of arrivals at specific African destinations. The assumption that 1 of 10 travelers is ≤18 years of age is consistent with the number of visits of young UK residents to African destinations in 2000. During that year, an estimated 138,000 persons in this age group accounted for 1,439,000 visits to Africa (*17*). The number of malaria cases in children per 10,000 visitors can be treated as a proxy. Using the number of child travelers as denominator, we calculated the rate of malaria cases acquired by children in the African regions and compared destination countries in Africa according to

malaria risk for young travelers. Denominator data did not account for time spent in the malaria-transmission area. We also used surveillance data to attempt to determine nationality or country of origin (ethnicity) of the children with malaria.

Results

Number of Cases

Of 17,009 reported malaria cases in children from the 11 industrialized countries studied, >75% were from only 3 countries (Table 1): France (n = 6,618), United Kingdom (n = 3,816; children ≤17 years of age), and United States (n = 2,614). The number of reported cases per year varied from 0 in Japan in 1996 to 1,096 in France in 1999. The number of cases registered in all contributing countries together was highest in 1999 (n = 2,233) and declined thereafter.

Among the different age groups, the largest overall percentage of cases occurred in those 15–17 years of age (18.1% of total cases) (Table 2). Analysis by age group showed heterogeneity between the countries. Japan and Australia showed high case rates; the 18-year age group accounted for almost 25% and 15% of all cases, respectively. Boys accounted for 55% of the total cases and predominated in all participating countries (data not shown).

Region of Malaria Acquisition

Of the 15,505 cases for which detailed data on country of acquisition were obtained, for all countries except Japan, >50% of cases were imported from Africa (Table 3); West Africa accounted for >50% of cases imported from Africa. Asia and Central and South America accounted for a small proportion of the imported malaria cases in children. Central and South America were responsible for a negligible number of infections, but in the United States, children accounted for 348 imported malaria cases (13% of all ma-

Table 1. Number of imported malaria cases in children in 11 industrialized countries, by year, 1992–2002*

Country	Year											Mean no. cases/y	Total no. cases
	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002		
Australia	162	144	138	111	183	160	147	158	119	107	75	137	1,504
Denmark	18	19	26	26	21	18	14	27	35	29	24	23	257
France	102	124	157	255	519	617	738	1,096	1,078	979	953	602	6,618
Germany	NA	41	75	65	96	77	57	93	68	113	72	76	757
Italy	NA	NA	NA	NA	NA	NA	93	72	69	96	77	81	407
Japan	5	4	5	9	0	4	1	6	5	2	4	4	45
Netherlands	13	22	17	43	41	36	37	35	79	57	40	38	420
Sweden	NA	NA	NA	NA	NA	31	21	31	16	20	36	26	155
Switzerland	37	51	37	34	41	49	32	35	34	36	30	38	416
United Kingdom	284	321	296	352	469	358	353	363	333	350	337	347	3,816
United States	159	206	173	215	293	333	253	317	227	263	175	238	2,614
Total cases	780	932	924	1,110	1,663	1,683	1,746	2,233	2,063	2,052	1,823		17,009

*All children were ≤18 years of age, except in the United Kingdom, where data were available only for children ≤17 years of age. NA, data not available.

Table 2. Number of imported malaria cases in children in 11 industrialized countries, by age group, 1993–2002

Country	Age group, y, no. (%)*							Total
	0–2	3–5	6–8	9–11	12–14	15–17	18	
Australia	131 (8.7)	143 (9.5)	139 (9.2)	174 (11.6)	233 (15.5)	456 (30.3)	228 (15.2)	1,504
Denmark†	26 (10.1)	44 (17.1)	47 (18.3)	37 (14.4)	30 (11.7)	55 (21.4)	15 (5.8)	257
France	1,149 (17.4)	1,247 (18.8)	1,054 (15.9)	1,042 (15.7)	920 (13.9)	887 (13.4)	319 (4.8)	6,618
Germany‡	112 (14.8)	123 (16.2)	120 (15.9)	117 (15.5)	97 (12.8)	142 (18.8)	46 (6.1)	757
Italy§	80 (19.7)	107 (26.3)	67 (16.5)	47 (11.5)	31 (7.6)	57 (14.0)	18 (4.4)	407
Japan	1 (2.2)	8 (17.8)	10 (22.2)	1 (2.2)	6 (13.3)	8 (17.8)	11 (24.4)	45
Netherlands	47 (11.2)	67 (16.0)	65 (15.5)	45 (10.7)	45 (10.7)	115 (27.4)	36 (8.6)	420
Sweden¶	12 (7.7)	20 (12.9)	26 (16.8)	30 (19.4)	24 (15.5)	32 (20.6)	11 (7.1)	155
Switzerland	60 (14.4)	81 (19.5)	72 (17.3)	65 (15.6)	55 (13.2)	60 (14.4)	23 (5.5)	416
United Kingdom	351 (9.2)	619 (16.2)	621 (16.3)	714 (18.7)	707 (18.5)	804 (21.1)	Not available	3,816
United States	333 (12.7)	463 (17.7)	409 (15.6)	386 (14.8)	399 (15.3)	463 (17.7)	161 (6.2)	2,614
Total cases	2,302 (13.5)	2,922 (17.2)	2,630 (15.5)	2,658 (15.6)	2,547 (15.0)	3,079 (18.1)	868 (5.1)	17,009

*Percentage of total cases in children <18 years of age (<17 years of age for United Kingdom).
†Data include 3 (1.2%) cases for which age was not specified.
‡Data from 1993–2002 only.
§Data from 1998–2002 only.
¶Data from 1997–2002 only.

laria cases in children [data not shown]). The predominant source of infections acquired in Asia was southern Asia (e.g., India, Pakistan, Sri Lanka) rather than Southeast Asia (e.g., Thailand, Indonesia, Vietnam, Malaysia, Philippines) (data not shown).

Case-Fatality Ratio

The case-fatality ratio for all countries was <0.4%; 3 countries (Italy, Sweden, and Japan) recorded no malaria-associated deaths in children during the period of observation (Table 4). Information about use of chemoprophylaxis in traveling children was limited. Among children with malaria, only 17.5% had taken chemoprophylaxis.

Plasmodium Species

Plasmodium species varied among the countries. The predominant species was _P. falciparum_, which accounted for 69.9% of all cases. The highest proportion of cases caused by _P. falciparum_ (83.1%) was in France (Table 5).

Return to Native Country

High-risk malaria destinations reflect the migrant population in the source country. For France, >2 million travelers visited Africa; the overall rate of malaria acquisition for children was 22/10,000 arrivals in Africa and 110/10,000 arrivals in considerable-risk countries within Africa. However, travelers to specific countries had a huge comparative risk. Children from France who visited the Comoros islands (n = 10,460) had a malaria attack rate of 1,251/10,000. From France, the Comoros islands are a recognized destination for visiting friends and relatives; no other country evaluated reported any malaria cases for travelers to the Comoros. In comparison, large numbers of travelers from France, probably tourists, visit Kenya (n = 527,880), where

the attack rate for the child travelers is 3.8/10,000 arrivals. The countries where children were most likely to acquire malaria were, in order of risk, Comoros (1,030 malaria cases/10,000 arrivals), Democratic Republic of Congo (778), Central African Republic (444), Guinea (308), Mali (203), Côte d'Ivoire (177), Congo (175), Nigeria (139), Bénin (134), Sierra Leone (127), Cameroon (109), Togo (102), and Ghana (100) and reflected the African nationality or origin of the immigrant communities in industrialized countries.

Discussion

During 1992–2002, >17,000 cases of imported malaria in children were reported in 11 industrialized countries in which malaria is not endemic. Of all cases in children with known place of disease acquisition, >75% were acquired in Africa, mainly West Africa. _P. falciparum_ was the dominant imported species; case-fatality ratio for all countries was <0.4%. Imported malaria in children is associated with travel, especially travel to visit friends and relatives, to high-risk malaria-endemic areas such as the Comoros islands and western and central African countries.

The strength of our study lies in the compilation of a large amount of data from national authorities, which enabled a global analysis. These data, coupled with data on arrivals in destination countries (*18*), enabled us to create a risk analysis for children traveling to malaria-endemic areas.

Limitations of our study include artifacts in malaria surveillance data and traveler statistics. Underreporting remains a problem in many countries (*19*), so our study could underestimate the true situation of imported malaria in children. Also, the quantity and quality of data received varied among countries and showed great heterogeneity despite

efforts to standardize reporting in Europe (*3*).

Surveillance systems and malaria case definitions differ; some countries rely on laboratories or clinicians or both for source data (*19*). One country had data for children up to only 17 years of age, some countries had data for only part of the requested period, and some countries used extrapolated data.

Nationality and ethnicity posed logistical problems for data analysis. In the absence of data on "reason for travel," we assumed that ethnicity represented the group who traveled to visit friends and relatives in their native country, which might not necessarily be true for countries such as the United States. The Figure shows the origin of the malaria patients rather than nationality, for which data are unavailable or unreliable. Information concerning the use of chemoprophylaxis is collected infrequently, if at all.

In some countries, e.g., Germany, the Netherlands, and Australia, notification systems changed during the study period (*20–22*). Our collated cases have also been influenced by traveler's choice of destination and use of preventive measures during travel, but other determinants included possible immunity or partial immunity of newly arrived immigrants to industrialized countries and number of travelers to malaria-endemic areas (*23*).

Statistics on traveler numbers, although imperfect, are the best available. To draw meaningful conclusions, we related numbers by destination country and source country to the corresponding visitation levels. Proxy data were used to estimate the percentage of young travelers to the various African destinations.

Data on arrivals need to be treated with caution because definitions, methods, and collection and compilation practices may differ from country to country. Many destination countries in Africa report arrivals by nationality and not by residency. With respect to malaria, method of reporting can imply that the number of visitors originating from the vari-

Table 3. Case rates for children in industrialized countries with malaria imported from Africa, 1992–2002*

Country of origin†	Western Africa No.	Western Africa Rate (95% CI)	Eastern Africa No.	Eastern Africa Rate (95% CI)	Central Africa No.	Central Africa Rate (95% CI)	Southern Africa No.	Southern Africa Rate (95% CI)	All African regions No.	All African regions Rate (95% CI)
Denmark	46	47.7 (34.9–63.6)	87	54.7 (43.9–67.5)	10	434.8 (208.5–799.6)	1	0.8 (0.02–4.5)	144	37.8 (31.9–44.5)
France	3,777	110.6 (107.1–114.2)	1,339	25.7 (24.4–27.1)	1,400	216.3 (205.1–228.0)	1	0.1 (0.001–0.7)	6,517	65.0 (63.4–66.6)
Germany‡	344	38.3 (34.3–42.5)	129	4.6 (3.8–5.4)	76	68.0 (53.6–85.2)	5	0.2 (0.07–0.5)	554	8.9 (8.2–9.7)
Italy§	323	40.2 (35.9–44.8)	24	2.0 (1.3–2.9)	31	39.5 (26.8–56.1)	1	0.3 (0.006–1.4)	379	15.2 (13.7–16.8)
Japan	8	7.0 (3.0–13.8)	3	1.1 (0.2–3.1)	4	102.6 (27.9–262.6)	1	0.5 (0.01–2.6)	16	2.6 (1.5–4.2)
Netherlands	172	35.3 (30.2–41.0)	32	0.6 (0.4 – 0.9)	51	103.9 (77.3–136.6)	1	0.1 (0.0–0.7)	256	13.9 (12.2–15.7)
Sweden¶	55	51.1 (38.5–66.5)	48	9.0 (6.6–11.9)	21	244.2 (151.2–373.3)	0	0.0 (0.0–1.7)	124	14.9 (12.4–17.8)
Switzerland	97	54.9 (44.5–67.0)	37	4.4 (3.1–6.1)	85	121.6 (97.2–150.4)	2	0.5 (0.06–1.9)	221	15.0 (13.1–17.1)
United Kingdom	1,749	177.3 (169.0–185.8)	406	9.7 (8.8–10.7)	91	82.3 (66.8–101.1)	14	0.4 (0.2–0.7)	2,260	26.3 (25.2–27.4)
United States	1,181	160.6 (151.6–170.1)	191	9.9 (8.5–11.4)	91	62.6 (50.4–76.8)	8	0.5 (0.2–1.0)	1471	33.7 (32.0–35.5)
Total	7,752	99.1 (96.9–101.3)	2,296	13.0 (12.5–13.5)	1,860	151.6 (144.8–158.6)	34	0.3 (0.2–0.5)	11,942	32.4 (31.8–33.0)

*Rates per 10,000 arrivals of children ≤18 years of age, except in the United Kingdom, where data were available only for children ≤17 years of age. Regions are classified according to United Nations World Tourist Organization (*18*). Denominators include only countries for which data were available. No data were available for arrivals to Gabon and Burundi for any country. For some African countries data are available for only some sources, e.g., arrivals to Sierra Leone available for only Denmark, United Kingdom; arrivals to Mozambique available for only United Kingdom, United States (details available on request). Western Africa comprises Benin, Burkina Faso, Cape Verde, Ghana, Guinea, Guinea Bissau, Côte d'Ivoire, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone, The Gambia, and Togo; Eastern Africa comprises Burundi, Comoros, Djibouti, Eritrea, Ethiopia, Kenya, Madagascar, Malawi, Mauritius, Mozambique, Reunion, Rwanda, Seychelles, Somalia, Sudan, Tanzania, Uganda, Zambia, and Zimbabwe; Central Africa comprises Angola, Cameroon, Central African Republic, Chad, Congo, Democratic Republic of the Congo (Zaire), Equatorial Guinea, Gabon; Sao Tome et Principe; and Southern Africa comprises Botswana, Lesotho, Namibia, South Africa, and Swaziland.
†No data available from Australia.
‡Data from 1993–2002 only.
§Data from 1998–2002 only.
¶Data from 1997–2002 only.

Table 4. Case-fatality ratios for children with imported malaria in 8 of 11 industrialized countries, 1992–2002*

Country	Total no. cases	No. deaths	Ratio (95% confidence interval)
France	4,893	10	0.20 (0.09–0.37)
Germany†	512	2	0.39 (0.05–1.40)
Italy‡	335	0	0.00 (0.00–0.89)
Japan	15	0	0.00 (0.00–18.10)
Sweden§	93	0	0.00 (0.00–3.17)
Switzerland	273	1	0.37 (0.01–2.02)
United Kingdom	2,502	5	0.20 (0.06–0.47)
United States	1,225	4	0.33 (0.09–0.83)

*All cases caused by *Plasmodium falciparum.* Children were ≤18 years of age, except in France and the United Kingdom, where data were available only for children ≤15 and ≤17 years of age, respectively. No data were available from Australia, Denmark, and the Netherlands.
†Data from 1993–2002 only.
‡Data from 1998–2002 only.
§Data from 1997–2002 only.

ous source countries is higher than the actual number (nationals residing abroad might not be included) and that the number of malaria cases/10,000 children is overestimated. Furthermore, several destination countries have small focal areas where malaria transmission occurs, yet the denominator estimate included travel to the whole country, which may falsely lower the malaria risk estimate.

Our finding of a high rate of *P. falciparum* cases in France is consistent with findings of several studies about imported malaria in children in France (*13,14,24,25*). Castéla et al. describe malaria in France as essentially imported from Africa (*13*). Eloy et al. found that 90% of the 60 children with malaria at the Versailles Hospital between January 1997 and December 2001 were of African origin and that 84% had *P. falciparum* malaria (*26*).

In Italy, between 1989 and 1997, a steady increase in the number of cases among foreigners in all age groups has been reported, while cases among Italian nationals have remained stable (*27*). In 2000, foreign nationals represent-

ed almost 73% of total imported malaria cases in all age groups; of these, 93% were African (*28*). In our study, 41% of children with malaria registered in Italy were of African nationality. Place of acquisition of *P. falciparum* infection was Africa for >93% of children; >75% of cases were acquired in West Africa.

In the United Kingdom in the 1970s, a large proportion of imported malaria cases were attributable to *P. vivax* and associated with a large number of immigrants from India and Pakistan. Since the 1980s, however, the situation of imported malaria in the United Kingdom has changed (*29,30*); the overall ratio of cases caused by *P. falciparum* to those caused by *P. vivax* has increased from ≈37% in the mid-1980s to 55% in the mid-1990s (*15*). Of the 3,816 cases registered in the United Kingdom during 1992–2002, 65.6% were caused by *P. falciparum*, and 27.1% by *P. vivax*. The higher number corresponds with >50% of persons from Africa, compared with 25% from the Indian subcontinent.

In the United States, cases were usually imported from Central America and Asia by immigrants, as well as by US travelers. However, as in other countries where traditionally *P. vivax* has been imported, cases acquired in Central and South America and Asia decreased and cases acquired in Africa increased (*31*). Dorsey et al. found that most patients who imported malaria to the United States had become infected while in Central and South America (38% [35% and 3%, respectively]), followed by West and East Africa (31% [22% and 9%, respectively]), and Asia (29% [Indian subcontinent, 20%; Southeast Asia, 9%]) (*32*).

In general our findings support those reported in the literature and show that Africa plays a key role in importing malaria in children to industrialized countries where malaria is not endemic. In our study, of all imported malaria cases in children, >70% were acquired in Africa.

Table 5. *Plasmodium* species causing imported malaria in children in 10 of 11 industrialized countries, 1992–2002*

	Total no. cases		Cases caused by known species, no. (%)			
Country	Including cases caused by mixed or unknown species	Excluding cases caused by mixed or unknown species	P. falciparum	P. vivax	P. ovale	P. malariae
Denmark	257	243	146 (56.8)	74 (28.8)	17 (6.6)	6 (2.3)
France	6,618	6,275	5,502 (83.1)	282 (4.3)	365 (5.5)	126 (1.9)
Germany†	757	685	512 (67.7)	140 (18.5)	16 (2.1)	17 (2.2)
Italy‡	407	402	335 (82.3)	34 (8.4)	19 (4.7)	14 (3.4)
Japan	45	40	15 (33.3)	19 (42.2)	5 (11.1)	1 (2.2)
Netherlands	420	349	237 (56.4)	74 (17.6)	23 (5.5)	15 (3.6)
Sweden§	155	142	93 (60.0)	33 (21.3)	9 (5.8)	7 (4.5)
Switzerland	416	368	273 (65.6)	71 (17.1)	13 (3.1)	11 (2.6)
United Kingdom	3,816	3,770	2,502 (65.6)	1,033 (27.1)	175 (4.6)	60 (1.6)
United States	2,614	2,397	1,225 (46.9)	982 (37.6)	65 (2.5)	125 (4.8)
Total	15,505	14,671	10,840 (69.9)	2,742 (17.7)	707 (4.6)	382 (2.5)

*All children were ≤18 years of age, except in the United Kingdom, where data were available only for children ≤17 years of age. No data were available from Australia.
†Data from 1993–2002 only.
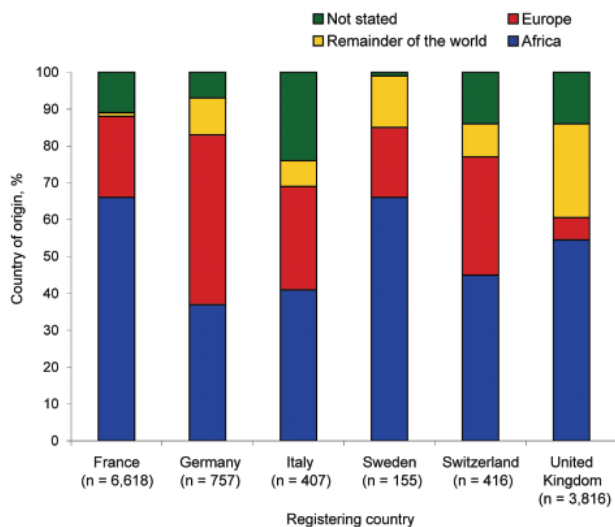‡Data from 1998–2002 only.
§Data from 1997–2002 only.

Figure. Country origin of 12,214 children with imported malaria in 6 industrialized countries, 1992–2002.

Imported malaria depends on the demographics of migrant populations and favored travel destinations of a country's settled immigrant community, such as the Comorean community in France or the Nigerian community in the United Kingdom. At high risk for malaria are settled immigrants and their children who visit friends and relatives in their country of origin. Many migrants seem to mistakenly believe that they retain their partial immunity against malaria parasites, but immunity usually wanes rapidly (within 6 months) in the absence of exposure to *Plasmodium*-infected mosquitoes, although some immunologic memory for malaria may exist (*8,15,33*). In addition, parents of children born and raised in an industrialized country in which malaria is not endemic may mistakenly believe their children have partial immunity (*15*). Use of chemoprophylaxis is recommended for all children who travel to high-risk malaria-endemic areas (*34*). Several studies have indicated, however, that correct use of and adherence to chemoprophylaxis is low (*13,16,35–38*).

Conclusions and Public Health Implications

Imported malaria in children is a complex problem that faces many challenges, including increasing global migrant and tourist travel; growing proportions of life-threatening falciparum malaria, combined with increasing resistance of malaria parasites to chemoprophylactic drugs; and lack of knowledge about and experience with imported malaria by physicians in industrialized countries where malaria is not endemic, which leads to delays in diagnosis and treatment of children with clinical malaria (*32,38*). The increasing proportions of *P. falciparum* cases are of relevance

because *P. falciparum* malaria carries the greatest risk for life-threatening illness. Increasing *P. falciparum* resistance to antimalarial medication endangers the effectiveness of antimalarial chemoprophylaxis; therefore, standard recommendations for chemoprophylaxis need to be continually updated. Specific research on malaria among children who visit their native countries is warranted. These children are the most likely persons to acquire malaria yet the least likely to use adequate prevention strategies. Culturally sensitive approaches to malaria risk awareness and prevention are urgently needed for schools, the travel industry, and community groups. Local health authorities in communities with large ethnic minorities, particularly of African origin, need to recognize the problem of imported malaria. Some worthwhile community-based programs have been initiated. We conclude that malaria prevention for children should be a task of primary care providers and should be subsidized for low-income travelers to high-risk malaria-endemic areas.

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Severe Dengue Epidemics in Sri Lanka, 2003–2006

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Recent emergence of dengue hemorrhagic fever in the Indian subcontinent has been well documented in Sri Lanka. We compare recent (2003–2006) and past (1980–1997) dengue surveillance data for Sri Lanka. The 4 dengue virus (DENV) serotypes have been cocirculating in Sri Lanka for >30 years. Over this period, a new genotype of DENV-1 has replaced an old genotype. Moreover, new clades of DENV-3 genotype III viruses have replaced older clades. Emergence of new clades of DENV-3 in 1989 and 2000 coincided with abrupt increases in the number of reported dengue cases, implicating this serotype in severe epidemics. In 1980–1997, most reported dengue cases were in children. Recent epidemics have been characterized by many cases in children and adults. Changes in local transmission dynamics and genetic changes in DENV-3 are likely increasing emergence of severe dengue epidemics in Sri Lanka.

Dengue viruses (DENVs) are mosquito-borne flaviviruses that each year infect millions of persons living in tropical and subtropical regions of the world. Several hundred thousand of these infections, especially in children, progress to a life-threatening disease known as dengue hemorrhagic fever (DHF). Dengue has emerged in many regions of the world and the number of cases and the range of the virus continue to increase every year (*1*).

The DENV complex consists of 4 distinct serotypes, designated DENV-1, DENV-2, DENV-3, and DENV-4. Infection with 1 DENV serotype is believed to provide long-term immunity to the homologous serotype but not to the other serotypes (*2*). Thus, persons can be infected with multiple serotypes during their lifetime. People with a repeat (secondary) DENV infection have a greater risk for DHF than persons infected for the first time, indicating that preexisting serotype cross-reactive immunity is a risk factor for severe disease (*3,4*). Furthermore, all 4 serotypes of DENV can cause DHF, but within each serotype some genotypes or clades within genotypes are linked to severe disease and others to mild disease (*5–7*).

Factors driving global emergence of dengue fever (DF) and DHF are complex and include viral and host factors as well as environmental changes that favor transmission. The epidemiology of dengue in Sri Lanka is particularly interesting because before 1989 all 4 serotypes were present and many repeat infections occurred, but few cases of DHF were documented (*8,9*). Incidence of DHF dramatically increased in 1989, and hundreds to thousands of cases of DHF have been documented every year since (*8*). Genetic studies with DENV-3 strains from Sri Lanka demonstrated that viruses isolated before and after emergence of DHF belonged to 2 distinct clades (DENV-3, genotype IIIA and IIIB, respectively), indicating that DENV-3 strain differences are likely to have contributed to emergence of DHF (*7*).

The magnitude of DF and DHF epidemics in Sri Lanka has continued to increase; 2 of the largest epidemics occurred in 2002 and 2004. We report results from dengue surveillance and virologic studies conducted during 2003–2006 in Sri Lanka. We also compare recent (2003–2006) and past (1981–1997) surveillance data and virus isolates to better understand factors driving emergence of severe disease in Sri Lanka.

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Materials and Methods

Sample Collection

Genetech Molecular Diagnostics Institute in Colombo, Sri Lanka, receives diagnostic specimens for dengue testing from clinics and hospitals in Colombo. This study used excess serum samples that remained after diagnostic testing. Only samples collected from patients with 1–4 days of fever were included in the study. All patient-identifying information was removed from specimens before their use in the study. The study was reviewed and approved by the Institutional Review Boards of the University of North Carolina, Chapel Hill, NC, USA, and the University of Sri Lanka, Peradeniya, Sri Lanka.

National Dengue Data Collected by Ministry of Health, 1996–2005

DF and DHF are reportable diseases in Sri Lanka. All practicing doctors treating dengue patients are expected to report cases to local health officers, who report cases on a weekly basis to the Central Epidemiology Unit of the Ministry of Health in Colombo. National data reported in this article are based on cases reported to the Central Epidemiology Unit. A special investigation form for collection of detailed information is sent out by the Central Epidemiology Unit for each reported case of DF or DHF to the reporting health office and the treating hospital. Age-specific disease information in this article was compiled from these special investigation forms.

Reverse Transcription–PCR for Detection and Serotyping of DENVs

Reverse transcription–PCR was performed by using the DV1 and DV3 primer set (*10*) and the ALD 1 and ALD 2 primer set (*11*) in 1 reaction. The DV primers amplify a 470-bp fragment of the nonstructural protein 3 (NS3) gene of all flaviviruses (*10*). The ALD1 and ALD2 primers amplify a 229–240-bp product from the 3′ untranslated region of all DENVs (*11*). The DV primers were not as sensitive as the ALD primers for detecting dengue infection. However, the 470-bp fragment amplified by the DV primers was used as the template in a second nested PCR to serotype the virus (*10*).

Isolation of DENV

For virus isolation, 15 μL of serum was mixed with185 μL of minimal essential medium containing 2% fetal bovine serum and added to C6/36 cells growing in 6-well tissue culture plates. The inoculum was incubated for 1 hour at 28°C before adding 2 mL of medium and incubating for 10 days in a CO_2 incubator at 28°C. Cells were tested for DENV by staining with monoclonal antibody 4G2, which binds to the envelope (E) protein of all 4 DENV serotypes.

Supernatants were harvested from positive wells and frozen as P1 DENV stocks.

Sequencing and Phylogenetic Analysis of DENV

The P1 stocks were used as a source of RNA for sequencing and genotyping viruses. Reverse transcription–PCR was performed with different primer pairs to amplify selected regions of the genome of DENV-1, -2, -3, or -4. For DENV-1, we amplified a 536-bp segment at the envelope-NS1 junction by using primers D1F 2034–2055 (5′-CCTTTTGGTGAGAGCTACATCG-3′) and D1R 2570–2551 (5′- ACACACACCCTCCTCCCA-TG-3′). For DENV-2, we amplified a 519-bp segment at the E-NS1 junction by using primers D2F 2050–2071 (5′-CCATTCGGAGACAGCTACATCA-3′) and D2R 2569–2548 (5′-GAGCCTTCTGGATAGCTGAAGC-3′). For DENV-3, we amplified a 1,057-bp segment encompassing part of the capsid (C) protein, the premembrane (preM) protein, and part of the E protein by using primers D3F 132–159 (5′- TCAATATGCTGAAACGCGT-GAGAAACCG-3′) and D3R 1189–1171 (5′- CTCCT-CAGGCAAAACCGCT-3′). For DENV-4, we amplified a 962-bp segment encompassing part of the C protein, preM protein, and part of the E protein by using primers D4F 137–162 (5′-TCAATATGCTGAAACGCGAGAGAACCG 3-′) and D4R 1099–1074 (5′-CCACTTCCTTGGCTGTTGT CTTGATC-3′).

Purified PCR products were sent to the University of North Carolina–Chapel Hill Genome Analysis Facility. Overlapping individual nucleic acid sequences were assembled by using VECTOR NTI (ContigExpress, Bethesda, MD, USA). Sequences were aligned and analyzed by using ClustalX (www.clustal.org), PAUP* (http://paup. csit.fsu.edu), PHYLIP (http://evolution.gs.washington. edu/phylip.html), and MEGA4 (www.megasoftware.net) software. All new virus sequences were deposited in GenBank (online Appendix Table, available from www.cdc. gov/EID/content/15/2/192-appT.htm) for virus strains and sequences used to create the phylogenetic trees.

Results

In January 2003, Genetech Molecular Diagnostics Institute in Sri Lanka began to test clinical specimens for DENV by PCR. Only samples collected from suspected dengue case-patients within the first 4 days of fever were tested. During 2003–2006, a total of 3,833 serum samples were received from hospitals and clinics. The number of samples tested ranged from 212 in 2003 to 1,686 in 2004 when Sri Lanka had a large DHF epidemic. Of the 3,833 samples, 930 (24%) were positive by PCR for DENV. On an annual basis, the proportion of positive samples was 39% in 2003, 22% in 2004, 18% in 2005, and 32% in 2006.

Comparison of Dengue Data Collected at Genetech with Nationally Reported Data

Figure 1, panel A, shows dengue cases reported to the Ministry of Health in Sri Lanka during 1980–2005. Cases reported before 1996 are based on passive surveillance and outbreak investigations conducted by the Ministry of Health. DF and DHF were designated as reportable diseases in 1996, and data obtained since 1996 are based on mandatory reporting. Although DENVs were common in Sri Lanka and persons there were exposed to multiple infections, severe disease was rare before 1989 (*8*). The 1990s were characterized by small but regular epidemics of severe disease (Figure 1, panel A) (*8*). In the period since 2000, the magnitude of the epidemics has increased further; particularly large epidemics occurred in 2002 and 2004 (Figure 1, panel A).

Most of the dengue samples tested at Genetech were received from private hospitals and clinics in Colombo. Because dengue is a reportable disease in Sri Lanka, physicians are expected to report cases to the Ministry of Health. We compared monthly dengue data reported to the Ministry of Health from Colombo and data collected at Genetech during January 2003–April 2006. Data from Genetech closely mirrored cases reported to the Ministry of Health, indicating that the laboratory at Genetech can serve as a sentinel site for monitoring DENV activity in the Colombo region (Figure 1, panel B). The peak number of cases observed at Genetech preceded reported peaks by ≈1 month (Figure 1, panel B); these cases from 2 sources were significantly correlated (correlation coefficient 0.80).

Circulating Dengue Serotypes

Of 930 PCR-positive samples collected during 2003–2006, we serotyped 605 samples by nested PCR. DENV serotypes 2 (40%) and type 3 (46%) were common, and serotypes 1 (7%) and 4 (7%) were rare. We examined the relative abundance of each serotype at monthly intervals during October 2003–September 2006 (Figure 2). DENV-2 and DENV-3 were the dominant serotypes throughout the study period. DENV-1 and DENV-4 were also regularly isolated but in low numbers. All 4 serotypes were detected in 2004 and 2006. In 2005, DENV activity was low and DENV-1 was not identified in samples. These results demonstrate that although all 4 serotypes cocirculate in Sri Lanka, DENV serotypes 2 and 3 are primarily responsible for clinically apparent cases.

Age Distribution of Dengue Case-Patients

When the age distribution of dengue-positive case-patients tested at Genetech during 2003–2006 was analyzed, we observed 2 peaks: the first in children <4 years of age and the second in adults 20–30 years of age (Figure 3). The peak of disease in adults was surprising because
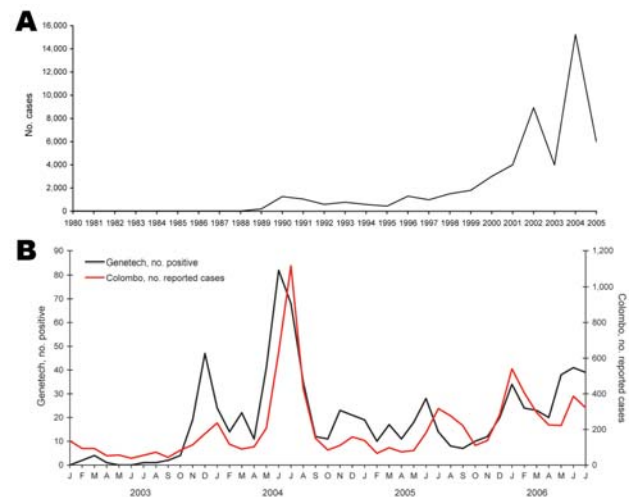

Figure 1. A) Dengue cases reported to the Epidemiology Unit, Ministry of Health, Sri Lanka (1981–2005). B) Comparison of monthly reported data for Colombo and Genetech for 2003–2006. Colombo data are based on cases reported to the Ministry of Health by hospitals and clinics within the Colombo Municipal Council. Genetech data are based on the number of PCR-positive cases detected each month.

previous studies indicated that dengue transmission in Colombo was high and most adults were likely to be immune to infection (*8*). To further evaluate the peak of disease observed in young adults, we examined the age distribution of case-patients reported to the Ministry of Health during 1996–2006. These data showed a striking change in age distribution of dengue case-patients over this 11-year period (online Technical Appendix, available from www.cdc.gov/EID/content/15/2/192-Techapp.pdf). Before 2000, one large peak of cases was observed in children and few cases were observed for adults. After 2000, two peaks of reported disease were observed for children and young adults. Moreover, the mean age of reported DF/DHF cases has increased from 15 in 1996 to ≈25 in 2006.
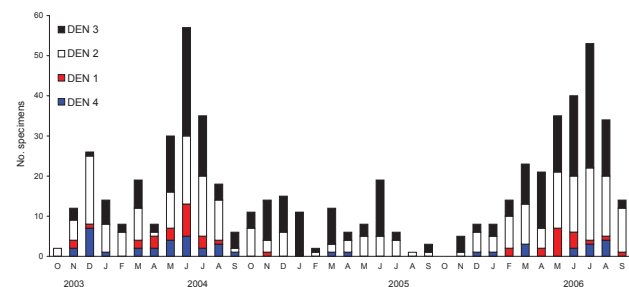

Figure 2. Relative abundance of dengue (DEN) virus serotypes in Sri Lanka. DEN-positive serum samples obtained from October 2003 through September 2006 were serotyped by reverse transcription–PCR.
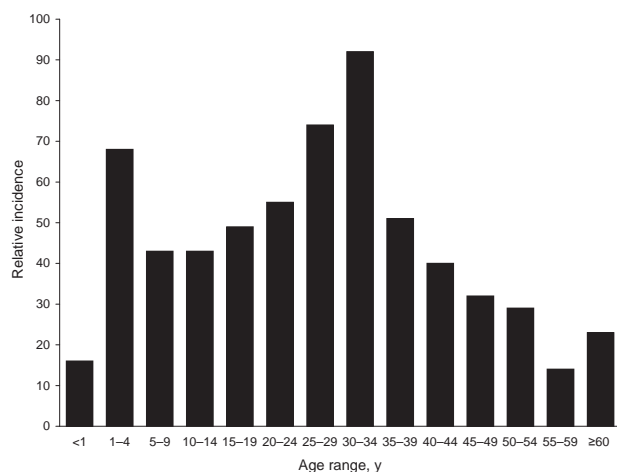
Figure 3. Clinically apparent dengue in different age groups in Sri Lanka, 2003–2006, Sri Lanka. Because true incidence data were not available, relative incidence of dengue infections by age cohort was estimated. We used Genetech data and known population of Colombo by age, to estimate relative incidence. The age group (≥60 years) with the lowest transmission rate was used as a referent for calculating the fold difference between each remaining cohort and the referent.

Phylogeny of DENVs

We reported that the DENV-3 strains isolated in Sri Lanka before and after the emergence of severe disease epidemics belonged to 2 distinct clades (7). To further characterize the DENVs responsible for recent epidemics in Sri Lanka, we isolated virus from serum samples collected in 2003 and 2004. The C6/36 mosquito cell line was inoculated by using serum samples from 220 samples that were positive by PCR for DENV. Virus was isolated from 181/220 specimens. The 181 isolates consisted of 18 DENV-1 strains, 76 DENV-2 strains, 64 DENV-3 strains, and 23 DENV-4 strains. To identify DENV genotypes that have been circulating in this country over the past 3 decades, we sequenced representative isolates from 2003 and 2004, as well as other isolates from Sri Lanka in our collection. All virus strains and sequences used for this analysis are listed in the online Appendix Table. When grouping each DENV serotype into different genotypes, we relied on the groups and nomenclature described by Rico-Hesse for the 4 serotypes (5). Genotypes are named on the basis of country of origin of the earliest isolates and not necessarily on current distribution of viruses.

Phylogeny of DENV-1 Strains Collected during 1983–2004

DENV-1 has been subdivided into 4 genotypes designated South Pacific, Asia, Thailand, and Africa/America (5). We evaluated the position of DENV-1 isolates from Sri Lanka within this established phylogeny. These isolates

used were obtained in 1983, 1984, 1997, 2003, and 2004. A 498-nt fragment from positions 2056 to 2554 (E/NS1 junction) was used to create a phylogenetic tree. Results demonstrate that the DENV-1 genotype circulating in Sri Lanka has changed over the study period. The 2 isolates from Sri Lanka obtained in 1983 and 1984 belonged to the South Pacific genotype (Figure 4). Sometime during 1984–1997, the Africa/America DENV-1 genotype became established on Sri Lanka and continued to circulate through 2004; the South Pacific genotype has not been detected during the past 8 years.

Phylogeny of DENV-2 Strains Collected during 1981–2004

DENV-2 has been subdivided into 4 genotypes designated Malaysian/Indian subcontinent, Southeast Asian, American, and West African (Sylvatic) (5). The Sri Lankan DENV-2 strains in our collection were isolated in 1981, 1982, 1983, 1984, 1985, 1989, 1990, 1996, 1997, 2003, and 2004. We sequenced the 239-nt fragment from positions 2311–2550 (E/NS1 junction) and generated a phylogenetic tree by using this sequence and existing sequences in Gen-Bank from representative DENV-2 strains. All DENV-2 isolates from Sri Lanka are closely related and belong to the Indian subcontinent/Malaysia genotype (Figure 5). Moreover, there is no evidence for the recent introduction of a DENV-2 strain from outside the island because the DENV-2 strains from Sri Lanka are more closely related to
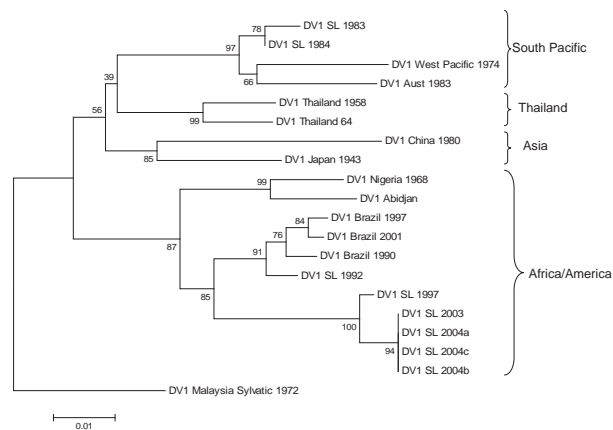

Figure 4. Phylogram of dengue serotype 1 viruses (DENV-1) from Sri Lanka (SL), 1983–2004, and other DENV-1 viruses. The tree is based on a 498-bp fragment for positions 2056–2554 coding portions of envelope protein and nonstructural protein 1. Evolutionary history was inferred by using the minimum-evolution method (12). Percentages of replicate trees in which the associated taxa clustered in the bootstrap test (1,000 replicates) are shown next to the branches (13). Phylogenetic analyses were conducted in MEGA4 (14). The tree was rooted by using a DENV-1 sylvatic strain. Classification and naming of different DENV-1 genotypes is based on the report by Rico-Hesse (5). Scale bar represents number of base substitutions per site.

Figure 5. Phylogram of dengue serotype 2 viruses (DENV-2) from Sri Lanka (SL), 1981–2004, and other DENV-2 viruses. The tree is based on a 239-bp fragment for positions 2311–2550 coding for amino acids at the envelope protein/nonstructural protein 1 junction. The tree was constructed as described in Figure 4 and was rooted by using a DENV-2 sylvatic strain. Classification and naming of different DENV-2 genotypes is based on the report by Rico-Hesse (*5*). Scale bar represents number of base substitutions per site.

one another than to any other DENV-2 strain used in this analysis.

Phylogeny of DENV-3 Strains Collected during 1983–2004

DENV-3 has been divided into 4 genotypes designated Southeast Asian/South Pacific (I), Thailand (II), Indian subcontinent (III), and American (IV) (*5,15*). Previous studies have demonstrated that all DENV-3 strains from Sri Lanka isolated in the 1980s and 1990s belong to Indian subcontinent genotype (III) (*7,15*). Within genotype III, DENV3 strains from Sri Lanka form 2 distinct clades linked to mild (IIIA) and severe (IIIB) disease epidemics on the island (*7*). DENV-3 IIIB viruses are most closely related to East African strains, which indicates that IIIB viruses linked to severe disease in Sri Lanka are likely to have been introduced from East Africa (*7*). In 1994, a DENV-3 genotype III from South Asia or East Africa was also introduced into Latin America, where it is now well established and responsible for severe disease epidemics (*7*).

Considering the backdrop of the recent expansion of DENV-3 genotype III viruses, we were interested in de-

termining the relationship of DENV-3 genotype III strains from Sri Lanka isolated in 2003 and 2004 to other DENV-3 genotype III viruses currently circulating in Africa, the Americas, and the Indian subcontinent. We sequenced the 966-nt fragment from positions 179–1144 (a portion of C, all of preM, and a portion of E) and created a tree by using our sequences and existing sequences in GenBank from representative DENV-3 genotype III strains. The DENV-3 sequences used were from isolates obtained in Sri Lanka in 1983, 1984, 1985, 1989, 1990, 1993, 1994, 1997, 1998, 2003, and 2004. As demonstrated previously by our group, DENV-3 genotype III consists of pre (IIIA)– and post (IIIB)–1989 clades from Sri Lanka, as well as Latin American and East African clades (Figure 6) (*7*). The DENV-3 strains from Sri Lanka isolated in 2003 and 2004 form a new, distinct clade that is closely related but distinct from the DENV-3 clade IIIB viruses that were isolated in the 1990s. This new 2003–2004 clade includes an isolate from 1993, which strongly suggests that the clade is derived from strains that have been on the island for some time.

Phylogeny of DENV-4 Strains Collected during 1978–2004

The phylogeny of DENV-4 has not been studied as extensively as the other serotypes. This serotype can be broadly separated into 2 genotypes designated Southeast Asian (I) and Indonesian (II) (*5*). The Southeast Asian genotype strains are primarily from Asia, whereas the Indonesian group has a broad distribution in Asia and the Americas. A 296-nt fragment from positions 787–1083 (preM/E junction) was used to create a phylogenetic tree. DENV-4 strains from Sri Lanka isolated in 1978 and in 2003–2004 group with the Southeast Asian genotype, which indicates that this genotype is established on the island (Figure 7). Two DENV-4 isolates from 1992 belong to the Indonesian genotype and likely represent a transient introduction (Figure 7).

Discussion

Although dengue has been a problem in the Indian subcontinent for at least the past 50 years, the disease and its viruses remain incompletely studied in the region (*16–18*). Over the past 2 decades, the epidemiology of dengue has changed and regular epidemics of DF and DHF have been reported in Sri Lanka, India, the Maldive Islands, Bangladesh, and Pakistan (*8,19–23*). We need a better understanding of the epidemiology of dengue in this region to develop and implement effective control programs and to most effectively use dengue vaccines that are currently in clinical trials.

We have investigated the emergence of DHF in Sri Lanka by analyzing samples sent for diagnostic testing to the Genetech Research Institute in Colombo. This insti-
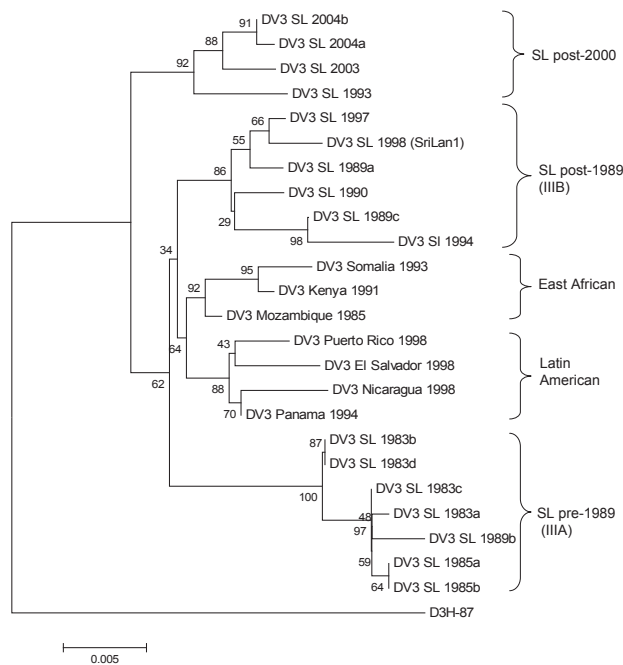
Figure 6. Phylogram of dengue serotype 3 (DENV-3) genotype III viruses from Sri Lanka (SL), 1981–2004, and other DENV-3 genotype III viruses. The tree is based on a 966-bp fragment for positions 179–1144 coding for a portion of the capsid protein, all of the premembrane protein, and a portion of the envelope protein. The tree was constructed as described in Figure 4 and rooted by using a DENV-3 genotype I virus (H87). Naming of the different groups within DENV-3 genotype III is based on the report by Messer et al. (7). Scale bar represents number of base substitutions per site

tute received 3,833 samples for testing during 2003–2006. On an annual basis, the proportion of samples positive for dengue was 39% in 2003, 22% in 2004, 18% in 2005, and 32% in 2006. The low proportion of positive samples in 2004 and 2005 compared with other years was unexpected because one would expect a greater proportion of positive cases during an epidemic year such as 2004. During the 2004 epidemic, there was widespread fear of dengue, and indiscriminate testing of fever cases is likely to have led to the overall lower proportion of positive cases.

We have demonstrated that all 4 serotypes cocirculate in Sri Lanka and were responsible for clinically apparent cases detected during 2003–2006. DENV types 2 and 3 were responsible for most human cases; types 1 and 4 were relatively rare. DENV types 2 and 3 native to the region may be more pathogenic than the other serotypes and thus may be recovered more frequently during human surveillance.

Viruses isolated during 1978–2004 were sequenced to understand the origin and evolution of DENV in Sri Lanka. For DENV-1, isolates obtained in Sri Lanka in the 1980s belong to the South Pacific genotype, whereas more recent isolates belong to the American/African genotype. These results indicate that the South Pacific genotype of DENV-1 was replaced after a new introduction of the American/African genotype of DENV-1. All DENV-2 isolates from Sri Lanka belong to a single (Indian subcontinent/ Malaysian) genotype. There is no evidence for introduction of DENV-2 from other areas because DENV-2 strains from Sri Lanka were more related to one another than to any other strain used in this analysis. All DENV-3 strains from Sri Lanka belonged to genotype III. However, in 1989 and again in 2000, the dominant clade of DENV-3 genotype III was replaced by a new clade of genotype III (7). In 1989, the lineage replacement was most likely caused by the introduction of DENV-3 from outside Sri Lanka. In 2000, the dominant lineage of DENV-3 was replaced by a previously rare lineage from Sri Lanka. The oldest (1978) and most recent isolates of DENV-4 belong to the Southeast Asian genotype, which indicates that this genotype is established on the island.

Perhaps the most striking feature of the epidemiology of dengue in Sri Lanka is the abrupt, stepwise increase in the number of severe disease cases in 1989 and again in 2000. In previous studies, we have highlighted the potential role of a shift in the circulating clade of DENV-3 from genotype IIIA to IIIB in this sudden emergence of severe disease in 1989 (7). Recent studies have also demonstrated that DENV-3 clade IIIB viruses replicate and disseminate better in the vector than clade IIIA viruses (24). This finding may explain the explosive spread of IIIB and closely related viruses within the region as well as into Latin America. The stepwise increase in cases after 2000 was accompanied by appearance of another clade of DENV-3 genotype III viruses that have replaced the clade IIIB viruses. Thus, evolution within DENV-3 genotype III continues to be linked to changes in disease severity in Sri Lanka. A
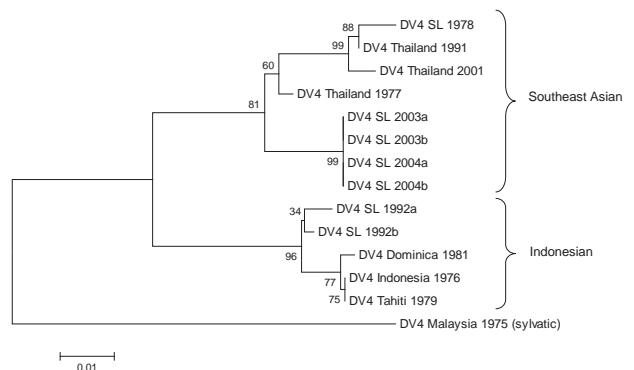

Figure 7. Phylogram of dengue serotype 4 viruses (DENV-4) from Sri Lanka (SL), 1978–2004, and other DENV-4 viruses. The tree is based on a 296-bp for positions 787–1083 coding for portions of premembrane and envelope proteins. The tree was constructed as described in Figure 4 and rooted by using a sylvatic DENV-4 strain. Classification and naming of different DENV-4 genotypes is based on the report by Rico-Hesse (5). Scale bar represents number of base substitutions per site.

similar phenomenon has also been reported by Bennett et al. (*25,26*). They compared data for 20 years from Puerto Rico for DENV-2 and DENV-4 and observed replacement of dominant clades by a previously rare lineage in the population or by viruses introduced from outside Puerto Rico. Clade replacement was linked to positive selection in the NS2A gene for DENV-4 and the E gene for DENV-2. Further studies are needed to assess if mutations in specific genes are also linked to emergence of new clades of DENV-3 in Sri Lanka.

When analyzing data collected at Genetech during 2003–2006, we observed a peak of dengue in young adults (20–30 years of age). Analysis of national data collected during 1996–2005 demonstrated that the disease peak in adults is a recent phenomenon and occurred after 2000. In areas with high transmission, where the virus has been historically established, most adults are likely to be immune because of childhood infections. The changing age structure may be indicative of the virus moving into new areas with many susceptible adults. During the large epidemics that occurred after 2000, many cases were reported from regions of the country where few cases have been reported (Epidemiology Unit, Ministry of Health, unpub. data). To better understand the molecular epidemiology and changing age distribution of dengue in Sri Lanka, laboratory-supported, population-based, active surveillance studies are needed.

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Dr Kanakaratne is a member of the Genetech Research Institute in Colombo, Sri Lanka. His research interests include study of emerging viruses and development of novel, cost-effective diagnostics for infectious diseases in developing nations.

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Seoul Virus and Hantavirus Disease, Shenyang, People's Republic of China

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An outbreak of hemorrhagic fever with renal syndrome (HFRS) occurred among students in Shenyang Pharmaceutical University in 2006. We conducted a study to characterize etiologic agents of the outbreaks and clarify the origin of hantaviruses causing infections in humans and laboratory animals. Immunoglobulin (Ig) M or IgG antibodies against Seoul virus (SEOV) were detected in the serum samples of all 8 patients. IgG antibodies against hantavirus were also identified in laboratory rats, which were used by these students for their scientific research. Phylogenetic analysis showed that partial small segment sequences recovered from humans, laboratory rats, and local wild rats belonged to SEOV. Hantavirus sequences recovered from humans and laboratory rats clustered within 1 of 3 lineages of SEOV circulating among local wild rats in Shenyang. These results suggest that the HFRS outbreak in Shenyang was caused by SEOV that was circulating among local wild rats and had also infected the laboratory rats.

Hantaviruses, members of the family *Bunyaviridae*, genus *Hantavirus*, cause 2 human zoonoses, hemorrhagic fever with renal syndrome (HFRS) in Asia and Europe and hantavirus pulmonary syndrome in North and South America (*1*). In their natural hosts, rodents of the families *Muridae* and *Cricetidae*, hantaviruses cause chronic infec-

tion with no apparent harm (*2,3*). HFRS has been recognized as a serious public health problem in China since 1955 (*4,5*). The disease is caused mainly by the *Hantaan virus* (HTNV), transmitted by the striped field mouse (*Apodemus agrarius*), and Seoul virus (SEOV), transmitted by the brown Norway rat (*Rattus norvegicus*) (*4,6*).

Transmission of hantaviruses among rodents and from rodents to humans generally occurs through inhalation of aerosolized excreta (*7*). HFRS outbreaks have occurred among farmers and workers during close contact with infected rodents in disease-endemic areas. Hantavirus infections have also occurred among technicians and researchers after handling laboratory rodents. The first report showed that contact with hantavirus-infected laboratory rats caused a HFRS outbreak among 13 doctors and 1 veterinarian at medical research institutions in Japan (*8*). Since 1975 and 1978, laboratory animal-associated HFRS outbreaks have been reported in several countries (*9–14*). Dozens of hantavirus infections in laboratory animals also occurred during the 1980s in China (*15*). Furthermore, 16 HFRS cases associated with laboratory rats occurred in 1983 in the Shanxi province (*16*). However, only a few reports have attempted to characterize the etiologic agents of the outbreaks and clarify the origin of hantaviruses causing infections in humans and laboratory animals (*9,17*).

Shenyang City (the capital of Liaoning Province) is located in northeastern China. Shenyang has always been one of the most seriously affected areas in China since the first outbreak of HFRS in 1958 (*5,18*). A total of 470 HFRS cases were reported in Shenyang in 2005; most of these cases occurred among farmers in the suburbs and the rural areas of Shenyang. Previous studies have shown the presence of 2 hantaviruses carried by rodents: HTNV, carried by striped field mice, and SEOV, carried by Norway rats

in Shenyang (*18*). Serologic and genetic analyses suggest that the HFRS outbreak was caused by transmission of SEOV, which was circulating among local wild rats; the wild rats passed the virus to laboratory rats, which then infected humans. Our study characterizes etiologic agents of these outbreaks among students and clarifies the origin of hantaviruses causing infections in humans and laboratory animals.

Materials and Methods

Patients and Serum Samples

HFRS cases were defined by a national standard of clinical criteria and confirmed by detecting antibodies against hantavirus in serum samples obtained in 2006. Serum samples were collected from patients with clinical signs of HFRS and sent to the Shenyang Center for Disease Control and Prevention (Shenyang CDC) for detection of hantavirus-reactive antibodies, and then to the Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention for further serologic and genetic characterization. Shenyang CDC conducted the HFRS epidemiologic studies. Information such as the date of onset of illness, fever, living conditions, history of exposure in dormitory and field, and clinical symptoms and signs was obtained and recorded.

Laboratory Rats and Mice

All laboratory rats (Wistar) and mice (BALB/c) housed in the same animal facility in a pharmaceutical laboratory building were obtained from the Laboratory Animal Center of Shenyang Pharmaceutical University and were sampled. These rodents were generally ≥6 months of age and had been in the animal facility for ≥1 month. Serum and lung tissue samples were collected from all laboratory animals, placed in vials, stored immediately at –196ºC, and transported to the laboratory for processing.

Trapping of Rodents

During 2006–2007, wild rodents were captured on the grounds of the animal facility in the pharmaceutical laboratory building, in the vicinity of the laboratory Animal Center of Shenyang Pharmaceutical University during 1 month after the outbreak in 2006, and in a major HFRS-endemic focus in the suburbs of Shenyang in the autumn of 2006 and the spring of 2007 using snap-traps baited with peanuts. Lung tissue samples were taken from dissected animals, placed immediately into vials and stored at –196ºC and, then transported to a laboratory for processing.

Serologic Assays

Human serum samples were tested for immunoglobulin (Ig) G and IgM antibodies against HTNV and SEOV by indirect immunofluorescent assay (IFA). Serum samples from laboratory rodents were tested for IgG antibodies to SEOV or HTNV. IgG and IgM IFAs were performed with HTNV (strain 76–118)– and SEOV (strain L99)–infected Vero E6 cells. Cells were spread onto slides, air-dried, and fixed with acetone. Samples were serially diluted in 2-fold steps in phosphate-buffered saline, starting with the initial dilution of 1:2, then added to the cells, and incubated for 90 min at 37°C. Slides were washed in phosphate-buffered saline and incubated with fluorescein isothiocyanate (FITC)–labeled rabbit antihuman IgG and IgM antibodies (Sigma, St. Louis, MO, USA), which are gamma-chain– and mu-chain specific, respectively, at 37°C for 30 min. For rodent samples, FITC-labeled goat antimouse or antirat IgG was used. IgG titers ≥40 and IgM titers ≥20 were considered positive.

Detection of Hantavirus Antigen

Viral antigens in the lung tissue (frozen sections) of rats and mice were detected by using indirect IFA as described previously (*19*), with rabbit anti-SEOV/L99 and HTNV/76–118 hantavirus antibodies and FITC-labeled goat antirabbit IgG (Sigma). Scattered, granular fluorescence in the cytoplasm was considered a positive reaction (Figure 1).

Reverse Transcription–PCR (RT–PCR) and Sequencing

Total RNA was extracted from rodent lung tissues by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions and subjected to RT-PCR for amplification of partial hantavirus small (S) segment sequences. cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (Promega, Beijing, China) in the presence of primer P14 (*20*). Partial S-segment sequences of SEOV (nt 620–999) were amplified from SEOV by using primers HV-SFO and HV-SRO for initial PCR (*21*), and primers SEO-SF and SEOV-SR for the second round of amplification (*22*). For amplification of partial S-segment sequence (nt 514–1026) from HTNV, the same primer pair HV-SFO/HV-SRO was used for initial PCR and the primer pair HSF /HSR was used for nested PCR (*22*).

The PCR products (380 bp and 513 bp, respectively) were gel-purified by using QIAquick Gel Extraction kit (QIAGEN, Beijing, China) according to the manufacturer's instructions and cloned into the pMD18-T vector (TaKaRa, Dalian, China). The ligated products were transformed into JM109-competent cells. DNA sequencing was performed with the ABI-PRISM Dye Termination Sequencing kit and an ABI 373-A genetic analyzer (Applied Biosystems, Carlsbad, CA, USA). At least 2 cDNA clones were used to determine each viral sequence. In case of discrepancy, a third cDNA clone was sequenced.
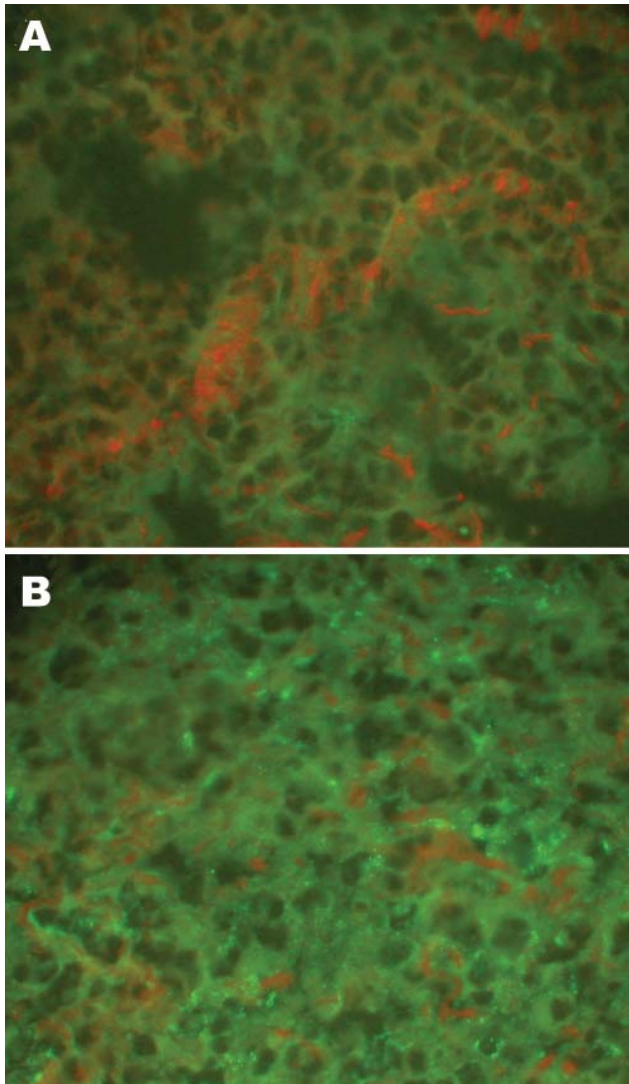
Figure 1. Detection of hantaviral antigens by indirect immuno-fluorescent assay. A) Hantaviral antigen- negative *Rattus norvegicus* lung tissue, detected with anti-L99 and 76-118 hantavirus sera. B) Hantaviral antigen-positive *R. norvegicus* lung tissue, detected with anti-L99 and 76-118 hantavirus antibodies. Magnification ×400.

Phylogenetic Analysis

The PHYLIP program package version 3.65 (http://evolution.genetics.washington.edu/phylip.html) was used to construct phylogenetic trees by using the neighbor-joining method with 1, 000 bootstrap replicates. Alignments were prepared with ClustalW version 1.83 (www.ebi.ac.uk/Tools/clustalw2/index.html). The nucleotide identities were calculated by using the DNAStar program (DNASTAR, Madison, WI, USA). For comparison, hantavirus sequences were retrieved from GenBank (www.ncbi.nlm.nih.gov/Genbank) (Figure 2).

Results

Patients and Survey Results

From March 8 through April 22, 2006, symptoms of hantavirus infection developed in 8 postgraduate students (5 men and 3 women), who studied at Shenyang Pharmaceutical University located in the center of the Shenyang. All patients met the national clinical criteria of HFRS, required hospitalization, and were treated in Shenyang Infectious Hospital. Fever, proteinuria, and mild hemorrhagic complications were observed in all patients, but without the distinct clinical stages seen in the severe form of the disease caused by HTNV (Table 1). Other clinical symptoms such as weakness, backache, nausea, vomiting, abdominal pain, eyeball pain, and hypotension were not observed.

The 8 students lived in different rooms in the 2 dormitories on the university campus. They had no history of exposure to wild rats in their rooms. Notably, all their roommates had been in good health. Further, the students neither performed field studies nor had a history of exposure to rats or mice in the field during the previous 6 months. All 8 students conducted their research in the same department and had direct contact with a colony of laboratory rats and mice in the animal facility in the pharmaceutical laboratory building. Hantavirus infection did not develop in any person who did not have direct contact with the laboratory rats and mice.

Serologic and Genetic Investigation of Patient Serum Samples

Serum samples from all 8 patients were collected at day 1 of hospitalization (2–4 days post onset of fever). Samples were tested for IgM and IgG antibodies by IFA using SEOV- or HTNV-infected cells (Table 2). All serum samples showed higher IgM and IgG titers in SEOV-specific IFA. In 6 of 8 sera, the IgG titers against SEOV were 4-fold higher; in the remaining 2 serum samples the titers against SEOV were 2-fold higher (Table 2). These results suggested that the HFRS cases were caused by SEOV.

Total RNA was extracted from all serum samples and analyzed by SEOV S-segment–specific or HTNV S segment–specific RT-PCR. Hantavirus genome sequences were amplified from 6 serum samples collected soon after the onset of disease by using SEOV S-segment–specific primers, not HTNV S segment–specific primers. That the HFRS cases were caused by SEOV was confirmed. Corresponding SEOV strains were designated ShenyangHu3, ShenyangHu4, ShenyangHu5, ShenyangHu6, ShenyangHu7, and ShenyangHu8.

Analysis of Laboratory Rats and Mice

Serum samples from all suspected laboratory rats and mice were tested for IgG antibodies against SEOV or
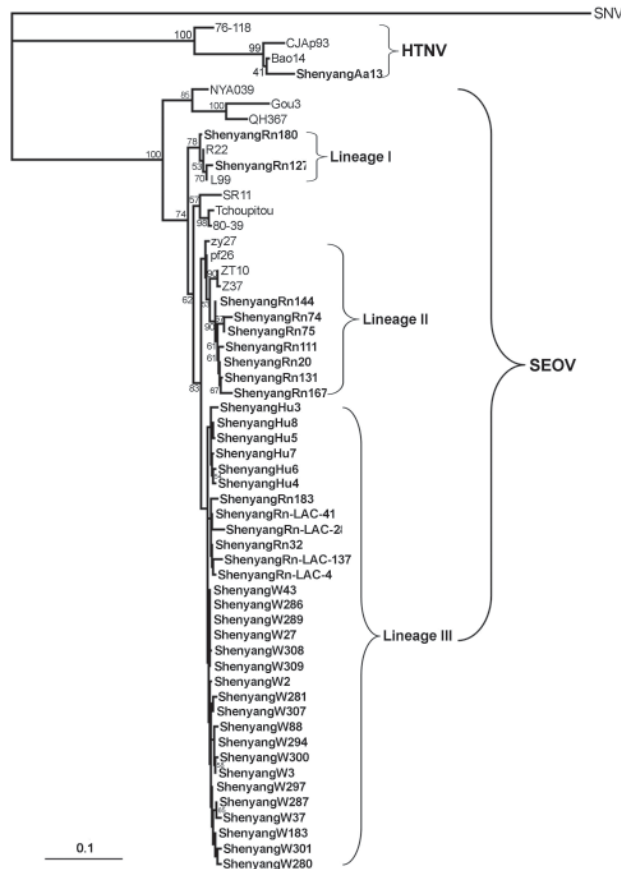
Figure 2. Phylogenetic tree of hantaviruses based on partial sequences of the small (S) segment (nt 600–999 for Seoul virus (SEOV) and nt 514–1026 for hantaan virus (HTNV). PHYLIP program package (3.65) was used to construct the phylogenetic trees by using the neighbor-joining (NJ) method and the maximum likehood (ML) with 1,000 replicates. The tree, constructed by using the ML method, had a similar topology as that constructed by the NJ method (data not shown). Bootstrap values were calculated from 1,000 replicates; only values >50% are shown at the branch nodes. The sequence of Sin Nombre virus (SNV) was used as an outgroup. Partial S-segment sequences recovered from 6 patient serum samples were designated ShenyangHu3, ShenyangHu4, ShenyangHu5, ShenyangHu6, ShenyangHu7, and ShenyangHu8. Sequences from *Rattus norvegicus* trapped in 2006 in the vicinity of the Laboratory Animal Center of Shenyang Pharmaceutical University were designated ShenyangRn-LAC-4, ShenyangRn-LAC-28, and ShenyangRn-LAC-41. ShenyangRn-LAC-137, from *R. norvegicus* and *A. agrarius,* trapped in 2006–2007 in the major hemorrhagic fever with renal syndrome–endemic focus in the rural areas of Shenyang were designated ShenyangRn20, ShenyangRn32, ShenyangRn74, ShenyangRn75, ShenyangRn111, ShenyangRn127, ShenyangRn131, ShenyangRn144, Shenyang Rn167, ShenyangRn180, ShenyangRn183, and ShenyangAa13), from hantavirus antigen–positive laboratory rats were designated ShenyangW–. Sequences obtained in this study are shown in **boldface**. The GenBank accession numbers of the other partial S segment sequences are SNV/NM H10 (L25748); HTNV/76–118 (M14626), HTNV/CJAp93 (EF208953), HTNV/Bao14 (AB127998); SEOV/NYA039 (EF210131), SEOV/Gou3 (AF288651), SEOV/QH367 (DQ081717), SEOV/SR11 (M34881), SEOV/Tchoupitoulas (AF329389), SEOV/80–39 (AY273791), SEOV/L99 (AF488708), SEOV/R22 (AF488707), SEOV/pf26 (AY006465), SEOV/zy27 (AF406965), SEOV/Z37 (F187082), and SEOV/ZT10 (AY766368). Scale bar represents genetic distance.

HTNV, and lung tissues were analyzed for the presence of hantavirus antigen by indirect IFA. Hantavirus antibodies were detected in 32 of 139 rats; the hantavirus antigen was detected in 26 of these 32 rats (designated ShenyangW–; Figure 2). Antibodies against HTNV or SEOV, or hantavirus antigen have not been observed in laboratory mice.

Rodent Trapping and Analysis

To investigate whether SEOV strains identified in the patients and laboratory rats originated in the local wild rodent population, 156 Norway rats (*R. norvegicus*) were trapped in the major HFRS endemic focus during the autumn of 2006 and the spring of 2007 in the vicinity of the Laboratory Animal Center of Shenyang Pharmaceutical University. Four of 156 wild rats were found to be positive for hantavirus antigen by IFA. Hantavirus S-segment sequences were recovered from these animals (corresponding hantavirus strains were designated ShenyangRn-LAC-4, ShenyangRn-LAC-28, ShenyangRn-LAC-41, and ShenyangRn-LAC-137). No rodents had been caught in the pharmaceutical laboratory building, suggesting that the laboratory animal infection occurred in the Laboratory Animal Center.

A total of 299 rodents (56 striped field mice [*A. agrarius*] and 243 Norway rats [*R. norvegicus*]) were captured in 2006–2007 during the major HFRS endemic focus in the rural areas of Shenyang, which is ≈15 km from the Laboratory Animal Center. Of these rodents, 11 Norway rats and 1 striped field mouse were found to be positive for hantavirus antigen by IFA. Hantavirus S-segment sequences were recovered from these animals (corresponding hantavirus strains were designated ShenyangRn20, ShenyangRn32, ShenyangRn74, ShenyangRn75, ShenyangRn111, ShenyangRn127, ShenyangRn131, ShenyangRn144, ShenyangRn167, ShenyangRn180, ShenyangRn183, and ShenyangAa13).

Genetic Analyses

Partial S-segment sequences were recovered from 6 patient serum samples, 19 laboratory rats (designated ShenyangW–, Figure 2); 15 wild Norway rats, and 1 striped field mouse trapped in the outbreak region. Genetic analysis showed that the partial S-segment sequences recovered from all humans, laboratory rats, and wild rats were very closely related to each other, with 95.6% to 99.8% sequence identity (online Appendix Table, available from www.cdc.

Table 1. Clinical symptoms and signs of HFRS patients in Shenyang, China, 2006*

Data	Patient no.							
	1	2	3	4	5	6	7	8
Sex	F	F	F	M	M	M	M	M
Age, y	24	24	25	24	24	24	24	29
Signs and symptoms								
Fever	+	+	+	+	+	+	+	+
Weakness	+	+	–	+	+	–	+	+
Headache	+	+	–	+	–	+	+	+
Backache	+	–	+	+	–	+	+	+
Eyeball pain	+	–	–	–	+	–	+	–
Nausea	–	–	–	+	–	–	+	+
Vomiting	–	–	–	–	–	–	+	–
Abdominal pain	–	–	–	+	–	–	–	–
Hemorrhagic complications	+	+	+	+	+	+	+	+
Oligouria	+	+	–	+	–	–	+	–
Proteinuria	+	+	+	+	+	+	+	+
Hypotension	+	–	–	+	–	–	–	–

*HFRS, hemorrhagic fever with renal syndrome.

gov/EID/content/15/2/200-appT.htm). These sequences have a higher level of identity to SEOV (85.5–99.2%) than to HTNV and other hantavirus types. Further comparison showed that the partial S-segment sequences recovered from human and laboratory rats were very closely related to each other, with 98.7% to 99.8% sequence identity. The 5% nucleotide divergence among hantaviruses carried by wild rats suggested that perhaps >1 genetic lineage of SEOV co-circulated in Shenyang. Notably, the sequences of hantaviruses carried by humans and laboratory rats were more closely related to those recovered from the wild rats trapped in the vicinity of the Laboratory Animal Center (ShenyangRn-LAC-4, ShenyangRn-LAC-28, ShenyangRn-LAC-41, and Shenyang-LAC-137). Moreover, these sequences also shared a higher homology with those recovered from the lung tissue samples that were collected from the wild Norway rats trapped in the major HFRS endemic focus in the rural areas of Shenyang (ShenyangRn32 and ShenyangRn180).

As expected, the partial S-segment sequence recovered from 1 striped field mouse was closely related to those from HTNV. The sequence showed especially high identity (99.0%) to strain Bao14 isolated from *A. agrarius* in Heilongjiang (*23*), which is also in northeastern China.

Phylogenetic Analyses

In the present study, phylogenetic analysis of partial S-segment sequences confirmed the molecular link between SEOV strains from patients, laboratory Norway rats, and the wild Norway rats trapped in the vicinity of the Laboratory Animal Center and the disease-endemic areas (Figure 2). As shown in Figure 2, all partial S-segment sequences from humans, laboratory rats, and wild rats fell into the SEOV genetic clade, well separated from other hantaviruses, thus indicating that the HFRS outbreak was caused by

SEOV. Notably, the partial S sequences from wild rats were divided into 3 lineages. The partial S sequences recovered from humans and laboratory rats formed 2 groups, and the sequences derived from the wild rats trapped in the vicinity of the Laboratory Animal Center formed another group. Together, these 3 groups formed a lineage that also included the sequences ShenyangRn32 and ShenyangRn183, which were recovered from the wild rats trapped in the major HFRS-endemic focus in the rural areas of Shenyang. This suggests that the HFRS outbreak had been caused by strains belonging to this particular lineage of SEOV.

Discussion

HFRS has been recognized as a serious problem in Shenyang since the first outbreak in 1958 (*18*). Despite comprehensive control measures, including vaccination, that have been carried out in the major endemic area of the city in the past several years, 361–630 HFRS cases have been reported annually from 2001 through 2005. Here we report the results of serologic and molecular epidemiologic

Table 2. Serologic analysis of samples from HFRS patients by indirect IFA, Shenyang, China, 2006*

Serum sample no.	IgM assay†		IgG assay†	
	SEOV	HTNV	SEOV	HTNV
1/06	40	20	640	160
2/06	40	20	320	80
3/06	40	–	320	80
4/06	20	–	320	160
5/06	40	–	320	80
6/06	40	20	160	20
7/06	40	20	320	80
8/06	40	–	320	160

*HFRS, hemorrhaghic fever with renal syndrome; IFA, immunofluorescent assay; Ig, immunoglobulin; SEOV, Seoul virus; HTNV, hantaan virus; –, could not be detected.
†Numbers represent the endpoint titers of anti-hantavirus (SEOV or HTNV) antibodies in the patients' serum samples.

investigation of a laboratory rats–associated outbreak of hantavirus disease involving 8 postgraduate students in Shenyang. The patients had clinical symptoms and biochemical findings typical of HFRS cases occurring in China. Serologic tests and the analysis of recovered hantavirus genome sequences showed that the outbreak was caused by a transmission of SEOV variants from the local wild Norway rats through the laboratory Norway rats to humans.

Serologic tests and phylogenetic analysis indicated that the HFRS cases were caused by the SEOV spread by laboratory rats. HFRS cases associated with laboratory-acquired infections have been reported in several countries (8–13,16). Notably, hantavirus infections were found to be more common in laboratory Norway rats than in mice and other laboratory animals (8,9,12–15). However, only a few investigations gave clear clues as to the origin of hantaviruses circulating in laboratory animals (9,17).

Previous studies have shown the presence of 2 hantaviruses carried by rodents: HTNV carried by the striped field mice and SEOV by the brown Norway rats in Shenyang (18). In the present study, serologic tests showed that all mouse serum samples were antihantavirus antibody-negative, and hantaviral antigens were not identified in the mouse lung tissues and HTNV-specific sequences were not detected in human serum samples. These results suggest that human infections were not caused by HTNV, although our data demonstrated that HTNV is circulating in *A. agrarius* in Shenyang. Both human and laboratory rat serum specimens were anti-SEOV antibody positive, which suggests that the infections were caused by SEOV. Due to the cross-reactivity of sera, exact serotyping for diagnoses of individual patients was not possible. Seroepidemiologic studies may sometimes misidentify the causative hantavirus if typing is based only on ELISA, IFA, or immunoblot analysis (24). Therefore, partial hantavirus S-segment sequences were amplified from the patient sera and laboratory rat lung tissues. All partial S-segment sequences recovered from 6 human and 26 laboratory rats belonged to SEOV; they were closely related to each other, and clustered together on the phylogenetic tree (Figure 2). These results confirmed that the HFRS outbreak in Shenyang was caused by SEOV and suggested the likely route of infection was from wild rats to laboratory rats and then to humans.

Analysis of wild rats trapped in the vicinity of human case-patients and the major HFRS epidemic focus allowed comparison of SEOV genome sequences in humans and rats (laboratory and wild). Phylogenetic analysis of the partial S-segment sequences indicated that 3 lineages of SEOV are co-circulating in wild rats in Shenyang (Figure 2). Notably, the sequences from patients and laboratory rats were clustered within 1 of these 3 lineages. Our results suggest that the viruses carried by the laboratory rats originated from

the prevalent SEOV strains circulating in wild Norway rats in this area, and then were transmitted to humans.

In conclusion, our study indicates that the HFRS outbreak was caused by SEOV circulating in local wild Norway rats through laboratory rats. Because hantavirus infection in wild Norway rats is frequent in most regions of China (5), this study reinforces conclusion that vigilance is needed to prevent laboratory-associated cases of hantavirus disease.

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Characteristics of 263K Scrapie Agent in Multiple Hamster Species

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Transmissible spongiform encephalopathy (TSE) diseases are known to cross species barriers, but the pathologic and biochemical changes that occur during transmission are not well understood. To better understand these changes, we infected 6 hamster species with 263K hamster scrapie strain and, after each of 3 successive passages in the new species, analyzed abnormal proteinase K (PK)–resistant prion protein (PrPres) glycoform ratios, PrPres PK sensitivity, incubation periods, and lesion profiles. Unique 263K molecular and biochemical profiles evolved in each of the infected hamster species. Characteristics of 263K in the new hamster species seemed to correlate best with host factors rather than agent strain. Furthermore, 2 polymorphic regions of the prion protein amino acid sequence correlated with profile differences in these TSE-infected hamster species.

Transmissible spongiform encephalopathy (TSE) diseases are infectious, fatal, neurodegenerative diseases of the central nervous system that affect a wide variety of mammals, including humans. In the past several decades, 3 new TSE diseases have been identified in different species: chronic wasting disease of deer and elk, bovine spongiform encephalopathy of domestic cattle, and variant Creutzfeldt-Jakob disease of humans. Thus, a better understanding of the process of cross-species transmission is needed.

Recognition of natural cross-species transmission is not straightforward. If a disease that crosses species has clinical or pathologic features similar to those of an already well-characterized TSE disease, it may not be recognized as a cross-species infection. Furthermore, some cross-species events involve slow processes in which the TSE agent

adapts over several passages before recognizable clinical disease occurs (1).

The amino acid sequence of the prion protein (PrP) is known to be an influential factor for cross-species transmission of TSE disease to a new host. Multiple single nucleotide polymorphisms resulting in amino acid changes that control susceptibility to TSE disease have been identified in sheep, cervids, humans, and transgenic mice (2–6). Similarly, incubation periods have also been correlated with nonsynonomous single nucleotide polymorphisms in the PrP gene. For example, 2 polymorphic residues at amino acids 108 and 189 are associated with either short (Leu108/Thr189) or long (Phe108/Val189) incubation periods in mice (7). Even a single point mutation in mice at amino acid position 101 has been shown to alter proteinase K–resistant prion protein (PrPres) deposition in brain, incubation periods, and host range (8).

In this study, we examined molecular and biochemical changes associated with cross-species transmission in 6 hamster species of the rodent subfamily *Cricetinae*. All animals were handled according to the National Institutes of Health guidelines and protocols approved by the Rocky Mountain Laboratories' (Hamilton, MT, USA) Institutional Animal Care and Use Committee.

Phylogenetic classification of these hamster subspecies is based on DNA sequences of mitochondrial cytochrome *b* gene and a portion of the NADH dehydrogenase 4 gene (9). These 6 species diverge into 3 genera, mainly *Cricetulus* (including Armenian and Chinese), *Phodopus* (including Djungarian and Siberian), and *Mesocricetus* (including Turkish and Syrian) hamster species (Figure 1). By inoculating each of these hamster species with a well-characterized, stable strain of Syrian hamster scrapie (263K), we were able to compare and analyze molecular and biochemical parameters of cross-species trans-
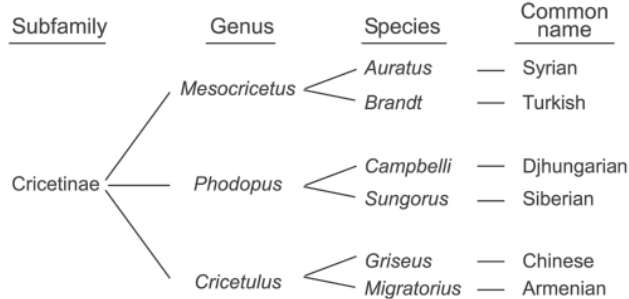
Figure 1. Taxonomic classification for 6 hamster species. Phylogenetically, these species are grouped into closely related taxonomic genera (9).

mission events. To identify the cross-species transmission event, we looked for recognizable features that may have emerged in the new host. We found that each new host species presented a profile unlike that of the original Syrian hamster host infected with 263K. These profile changes correlated with unique PrP amino acid sequences within the 6 hamster species. This finding suggests that host PrP sequences can change the phenotype presentation of the agent in the host and could thereby confound identification of cross-species transmission events.

Materials and Methods

Incubation Periods and Titers for Passaged 263K

The original source of 263K hamster agent came from Kimberlin et al. (10) and was passaged 3 times in Syrian hamsters at Rocky Mountain Laboratories. For cross-species transmissions, also referred to as first passage, weanling hamsters from each of the 6 hamster species were intracranially inoculated with 263K stock at a titer of 2×10^9 lethal dose for 50% per 50 μL of a 1% brain homogenate. Clinically ill hamsters from each species were killed, and 1% brain homogenate was passaged intracranially into weanling hamster recipients of the same species (second passage). The process was repeated for a third passage. Brain homogenate from clinically ill third-passage hamsters was inoculated intracranially back into Syrian (back-passaged) hamsters.

Each hamster was killed when it had lost ≈30% of its body weight and was no longer able to remain upright and feed itself. We determined endpoint titrations for 263K, first, second, and third hamster passage inocula used in these experiments for all species (except Chinese hamsters) by preparing sequential 1:10 dilutions of a 1% brain homogenate to 10^{-8} or 10^{-10}. Dilutions were then injected intracranially into 6–8 hamsters for each dilution, and titers were determined as described (Figure 2) (11).

Immunoblot Analysis of Proteinase K–Sensitive Prion Protein and PrPres by Western blot

Proteinase K (PK)–sensitive prion protein (PrPsen) and PrPres were prepared as previously described (12,13). Samples were frozen at –20º C until they were subjected to electrophoresis on a 16% sodium dodecyl sulfate–polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). Immunoblots were probed by using polyclonal antibody R30 to PrP (89–103 in Syrian hamsters) (14,15), which recognizes PrP from each of the 6 species. Blots were developed by using either enhanced chemiluminescence or enhanced chemifluorescence according to manufacturer's instructions (Amersham-Pharmacia, Uppsala, Sweden). Enhanced chemifluorescence blots were scanned by using a STORM fluorescent detection system (Amersham-Pharmacia) as described previously (16).

Sensitivity of Proteinase K

To demonstrate PrPres sensitivity to PK, we adjusted 20 μL of a 20% (wt/vol) third-passage brain homogenate in 0.01 M Tris, pH 7.3, from each species to 100 mmol/L Tris HCl, pH 8.3, 1% Triton X-100, and 1% sodium deoxycholate. Samples were treated with 25 μg/mL, 100 μg/mL, 400 μg/mL, or 1,600 μg/mL PK in a total volume of 35 μL and incubated at 37º for 1 h. The reaction was stopped by adding 2 μL of 0.1 M phenylmethylsulfonyl fluoride and placed on ice for 10 min. Samples were then mixed in equal volumes with 2× sample buffer, boiled 5 min, and subjected to electrophoresis on sodium dodecyl sulfate–polyacrylamide gels. PrP bands were quantitated as described above.

PrP Gene Sequencing

To sequence the PrP gene open reading frame (ORF), we based primers on published regions of sequence homology between the Syrian, Armenian, Chinese hamster; rat; and mouse
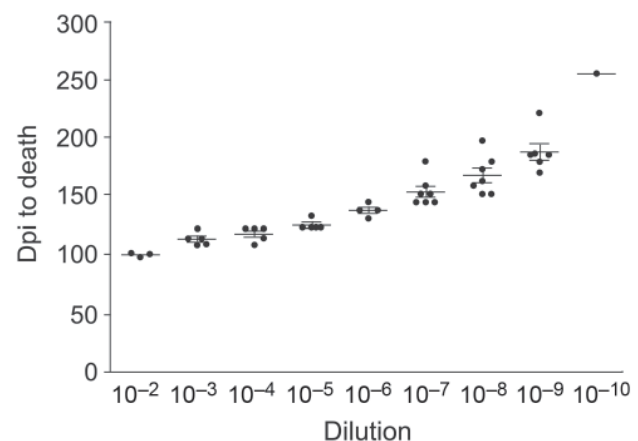

Figure 2. Example of titration curves for all homogenates titered. The curve shown is Djungarian second-passage brain homogenate with 3–7 hamsters per dilution. Error bars indicate SEM. Dpi, days postinoculation.

PrP genes. Primers 7F, S/O(–1), C(–1), S/T(+2), C/O(+2), and 1R are located outside the ORF. The following primers were used: 7F-5′-GCCTTGTTCTTCATTTTGCAGA-3′, S/O(–1) 5′-TCATTTTGCAGATCAGCCATC-3′, C(–1) 5′-TCATTTTGCAGATTAGCCAT-3′, S/T(+2) 5′-GTACAAGCAGGGAGGCTTCCTTC-3′, C/O(+2) 5′-GTACAAGCAGGGAGGCTTCCCTC-3′, and 1R 5′-ACCCCTCCCCCAGCCTAG-3′.

Immunohistochemistry

Immunohistochemistry procedures were conducted as previously described (*12*). PrPres was detected by using R30 anti-PrP antibody (residues 89–103).

Results

Incubation Periods and Titers

Incubation periods and infectivity titers are among the criteria used to define TSE strains (*17,18*). Therefore, we compared incubation periods and infectivity titers for 263K in Syrian hamsters with those observed for the 5 other hamster species. Cross-species transmission of Syrian-derived 263K to Turkish hamsters, both members of the genus *Mesocricetus* (Figure 1), had similar incubation periods (Table). Transmission of 263K to hamsters of the genus *Phodopus* (Djungarian and Siberian) had incubation periods similar to each other but different from those of the *Mesocricetus* hamsters. Although Chinese and Armenian hamsters each belong to the genus *Cricetulus*, after inoculation with 263K, their incubation periods differed from each other and from those of all the other species. In most instances, we observed prolonged incubation periods when Syrian 263K was inoculated into the new hosts. Because all the hamsters received the same inoculum at passage 1, this finding likely reflects the species barrier between the hosts (*19*) rather than inoculum titer.

Two additional passages from a donor to a recipient of the same species showed that incubation periods in most species differed considerably from those of the original Syrian hamsters (Table). Incubation periods for the new species decreased and then became stable between second and third passage and were considerably different from those of the original Syrian hamsters (Table). Incubation periods for Chinese hamsters were still decreasing between second and third passages. Because a fourth passage was not performed for Chinese hamsters, whether third passage reflects the stable incubation period is unknown. Thus, for Djungarian, Siberian, and Armenian hamsters, evidence was strong for unique incubation periods in the new species by second passage after inoculation with Syrian 263K agent.

To determine whether incubation period differences for each species resulted from differing infectivity concentration in the inocula, we used endpoint titration to determine the brain titers after first, second, and third passages (Table). These determinations were not conducted for Chinese hamsters because the long incubation periods would require several additional years. When comparing first-passage titers with titers in Syrian hamsters infected with 263K, the only decrease in infectivity titer was seen in Armenian hamsters (Table). When comparing second-passage titers with titers in Syrian hamsters infected with 263K, we observed a minimal decrease in Turkish, Siberian, and Armenian hamsters. We observed no notable changes in titers among third passage animals of all 6 species, which suggests stable titers in each species by third passage.

Additional evidence for adaptation of 263K agent to the new host species was obtained by infecting Syrian hamsters with brain homogenates from third-passage hamsters. If the agent had adapted to the new species, we would expect to see differences in incubation periods. In contrast, in the absence of adaptation, we would expect reversion back to the characteristic 263K Syrian incubation period. The only hamster for which third-passage brain homogenate resulted in a decrease in incubation period was the Chinese hamster. Even so, at 168 days this incubation period still differed from the characteristic 80-day incubation period for Syrian hamsters. These results indicate that the 263K agent adapted to the new host and that each 263K-infected hamster species has a unique incubation period (Table).

Glycoform Profiles

PrPres glycoform profiles are another criteria used to

Table. Average incubation period for 263K scrapie*						
	Cross-species 263K		Second passage		Third passage		Back passage‡
Hamster species†	Dpi ± SD	Titer/50 μL (1% BH)	Dpi	Titer/50 μL (1% BH)	Dpi	Titer/50 μL (1% BH)	Dpi
Syrian	85	2×10^9	ND	ND	ND	ND	79 ± 5
Turkish	91 ± 4.9	$1 \times 10^{8.9}$	103 ± 7.2	$1 \times 10^{6.7}$	85 ± 7.6	$1 \times 10^{8.25}$	76 ± 0
Djungarian	155 ± 23.4	$1 \times 10^{8.4}$	100 ± 9.0	$1 \times 10^{9.6}$	99 ± 7.2	$1 \times 10^{9.7}$	97 ± 7
Siberian	148 ± 26.5	$1 \times 10^{8.3}$	117 ± 9.9	$1 \times 10^{7.75}$	114 ± 8.3	$1 \times 10^{8.1}$	128 ± 17
Chinese	372 ± 29.1	ND	233 ± 17.9	ND	207 ± 15.2	ND	168 ± 7
Armenian	188 ± 10.4	$1 \times 10^{7.35}$	156 ± 4.8	$1 \times 10^{7.75}$	145 ± 7.5	$>1 \times 10^{8.5}$	145 ± 18

*Dpi, days postinoculation; ND, not done; BH, brain homogenate.
†16 hamsters/species.
‡In Syrian hamster.

differentiate TSE strains (*20–22*). Therefore, we compared PrPres glycoform profiles from each of the 6 hamster species at each passage.

When Western blotting was used to compare percentages between the 3 PrPres bands (Figure 3, panel A), the data clearly showed 2 different PrPres glycoform profiles. The Turkish hamster PrPres glycoform profile shared similarities with that of both Syrian and Chinese hamsters. Siberian, Djungarian, and Armenian hamsters shared a second PrPres glycoform profile. PrPres glycoform patterns from Turkish, Chinese, Siberian, and Djungarian hamsters fluctuated noticeably over the 3 passages, which suggests a lack of stability while adapting to the new species. In contrast, PrPres glycoform patterns from Armenian hamsters did not fluctuate over the 3 passages, which suggests a stable strain in Armenian hamsters (Figure 3, panel A).

We also injected brain homogenates derived from the third-passage hamsters back into Syrian hosts. We found that glycoform patterns in the Syrian recipients were the same as those ordinarily associated with Syrian hamsters (Figure 3, panel B), which suggests, as with incubation periods, that the host had a predominant influence over glycoform patterns.

PrPsen

To investigate the possibility that differences in PrPres glycoform profiles were reflections of different PrPsen characteristics in the various hamster species, we analyzed PrPsen profiles by using Western blot. No differences were found in expression levels or banding patterns among the 6 hamster species (Figure 4, panel A). Bands detected at 37 kDa were proven to be PrPsen because they were competed out (protein signal disappeared) when we preincubated the antibody to PrP with a synthetic peptide specific for the PrP epitope. Bands >37 kDa did not compete out (Figure 4, panel B). All PrPsen samples were PK sensitive (data not shown).

PK Resistance

PrPres resistance to PK digestion has also been used to differentiate TSE strains (*23–25*). When we compared PrPres resistance to PK from 263K Syrian and third-passage Turkish, Armenian, Chinese, Siberian, and Djungarian hamsters, we found no differences in sensitivity to PK. All samples retained equivalent PrPres signals on Western blots after treatment with 25 and up to 1,600 μg/mL PK (data not shown).

Immunohistochemical Findings

Differentiation of TSE strains has also been based on regional distribution of PrPres and microscopic lesions in brains of infected individuals (*18*). Therefore, we studied lesion profiles in brain from 5–8 hamsters of each species
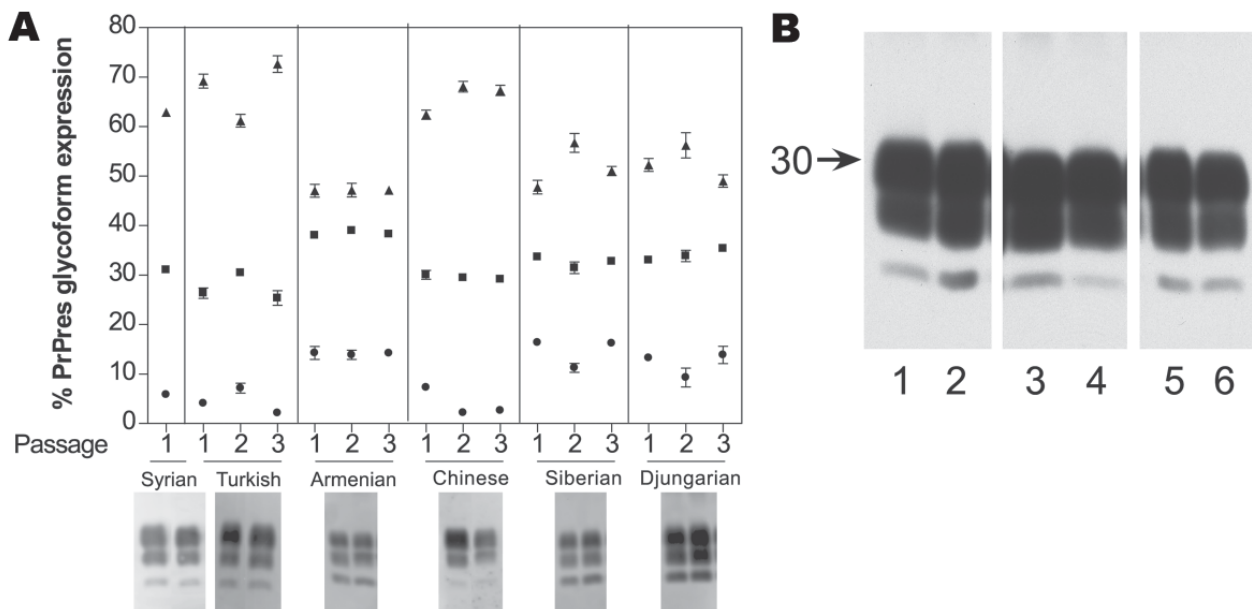

Figure 3. A) Proteinase K–resistant prion protein (PrPres) glycoform profiles for 6 hamster species for each of 3 successive passages: 1, initial cross-species passage; 2, second intraspecies passage; 3, third intraspecies passage. Each passage represents 6 different animals, each quantified 6–8 times. Each lane had 0.5 mg tissue equivalents per lane. ●, percentage of unglycosylated band; ■, partially glycosylated band; ▲, fully glycoslyated band. Western blot representation of glycoform for each species visualized using R30 and enhanced chemifluorescence. Error bars indicate SEM. B) Serially passaged 263K scrapie from 5 hamster species passaged back to Syrian hamster. Western blot analysis of clinically ill Syrian hamster infected with Syrian 263K or 263K passaged 3 times through the new hamster host. Syrian hamster inoculated with brain homogenate from the following hamsters: lane 1, Syrian 263K; 2, Turkish; 3, Chinese; 4, Armenian; 5, Djungarian; 6, Siberian. Tissue equivalents: lane 1, 0.5 mg; lanes 2, 5, and 6, 0.4 mg; lane 3, 0.7 mg; and lane 4, 0.9 mg.
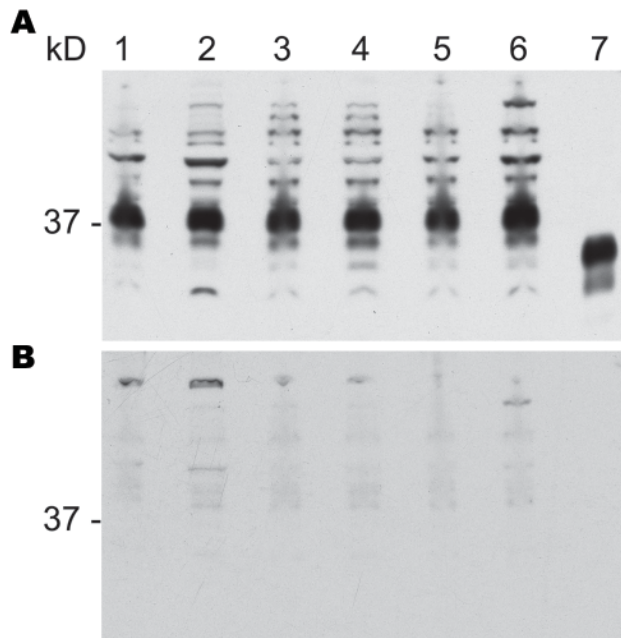
finding was not surprising. In the other 4 species (Djungarian, Siberian, Armenian, and Chinese), PrPres distribution in the thalamus was increased (region 6) over that in the same region for Syrian hamsters. Further changes were seen in Armenian hamsters; PrPres distribution was increased in the spinal cord (region 5) and olfactory bulb (region 10). PrPres distribution patterns in Chinese hamsters diverged from those in the Syrian hamsters in almost every region except the spinal cord (region 5). In all 6 species, including our original Syrian hamster species, unique pathologic phenotypes developed. Immunohistochemical and glycoform
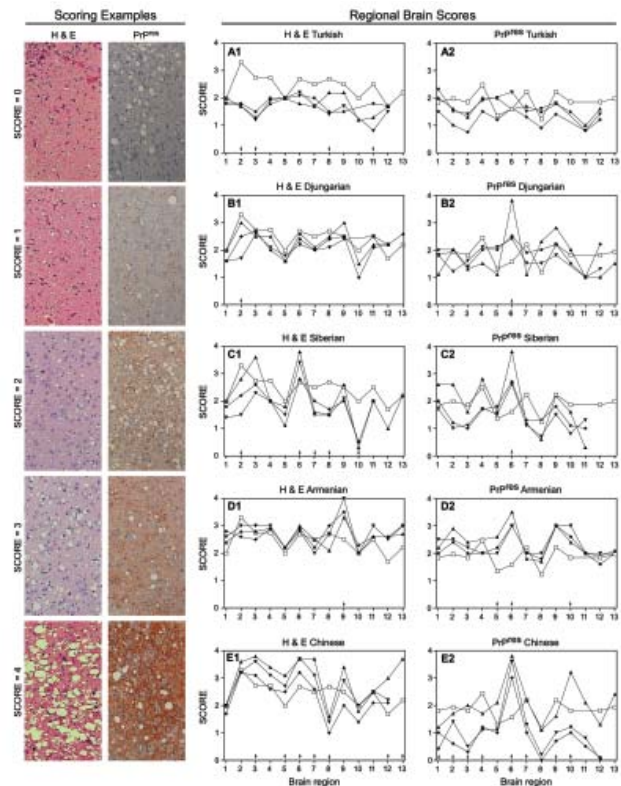

Figure 4. Proteinase K–sensitive prion protein (PrPsen) Western blot analysis from 6 hamster species performed with A) polyclonal antibody R30 (89–103) or B) R30 preincubated with peptide to prion protein 89–103. Hamster species: lane 1, Syrian; lane 2, Turkish; lane 3, Djungarian; lane 4, Syrian; lane 5, Chinese; lane 6, Armenian. Lane 7, proteinase K–resistant prion protein (PrPres) from 263K Syrian hamsters. 0.8 mg tissue equivalents per lane; 37 kDa indicated.

at each passage. We scored 13 areas from 0 to 4 (none to the highest degree of PrPres distribution or lesions) (Figure 5, Scoring Examples). Averages for each brain region were scored and compared (Figure 5, Regional Brain Scores). A score change >1 between species or passages was considered a notable change for that region (*26*).

Microscopic lesion profiles (Figure 5, panels A1–E1) from the 3 passages for each species were compared with those of the 263K Syrian hamsters (stained with hemotoxylin and eosin [H&E]). We found that Djungarian hamster profiles were most similar to 263K Syrian hamster profiles. Second-passage Armenian hamsters had increased vacuolation in the cortex; Turkish, Siberian, and Chinese hamsters differed in multiple regions. Lesion profiles for the 3 passages in each of the new hamster host species, such as Chinese hamsters (Figure 5, H&E Chinese) regions 2 and 3, did not necessarily correlate with PrPres distribution (Figure 5, panel PrPres Chinese) within that host.

When comparing the PrPres deposition profile (Figure 5, PrPres panels [right side]) at each of the 3 passages to the Syrian PrPres deposition profile, we found that the profiles of the Turkish hamsters were the most similar. Because Syrian and Turkish hamsters are closely related phylogenetically and share similar PrPres glycoform patterns, this

Figure 5. Scoring examples: Hematoxylin and eosin (H&E)–stained and proteinase K–resistant prion protein (PrPres) scores from 0 to 4. A score of 0 means no vacuolation or PrPres distribution; a score of 4 means the highest degree of vacuolation and PrPres distribution in that region. Panel H&E score: 0, Chinese hamster olfactory bulb; 1, Syrian hamster spinal cord; 2, Armenian hamster caudate putamen; 3, Armenian hamster cortex; 4, Syrian hamster thalamus. Panel PrPres score: 0, Chinese hamster superior colliculus; 1, Syrian hamster posterior colliculus; 2, Chinese hamster brain stem; 3, Djungarian hamster posterior colliculus; 4, Chinese hamster thalamus. Regional Brain scores: all hamsters were compared with 263K Syrian hamster (□) shown in each panel A1–E1 and A2–E2. Lesion and PrPres profiles of 263K scrapie-infected hamster from each of the 5 new hamster species. Each point represents the average from 6 different animals scored in the following areas: 1, cerebellum; 2, posterior colliculus; 3, superior colliculus; 4, brain stem; 5, spinal cord; 6, thalamus; 7, hypothalamus; 8, hippocampus; 9, cortex; 10, olfactory bulbs; 11, caudate putamen; 12, septal nucleus; 13, tegmentum. ▲, first passage; ▼, second passage; ♦, third passage.

profiles for 2 of the 3 genera were independently unique, which suggests that similar host factors within those genera influenced these TSE characteristics.

To determine the respective contributions of the agent and host in immunohistochemical patterns, we injected brain homogenate from third-passage hamsters back into Syrian hamsters. When patterns were compared with those of 263K Syrian hamster, only a few differences from 263K Syrian were observed. Specifically, less PrPres was deposited in the posterior colliculus (region 2) in Syrian hamsters inoculated with brain homogenate from Chinese hamsters, and more PrPres was deposited in the thalamus (region 6) of Syrian hamsters inoculated with brain homogenate from Djungarian hamsters (Figure 6, panel A). We also observed a decrease in vacuolation in the hippocampus (region 8) and cortex (region 9) of Syrian hamsters inoculated with brain homogenate from Chinese hamsters (Figure 6, panel B). For all other hamster species inocula, no remarkable differences from 263K Syrian hamster were noted. Therefore, the host appeared to play an important role in determining immunohistochemical patterns. It is not known whether the differences seen in the Chinese and Djungarian hamsters are due to donor species effect (*27,28*), whereby a temporary change occurs as an agent is passed through a new host species, or whether selection of a new strain has occurred. To answer this question, a second passage through Syrian hamsters is needed. These results suggest that although 263K agent was substantially altered by passage through the new hamster species, in general it was not able to impart molecular characteristics like glycoform or the pathologic patterns established in the new species back to the Syrian hamsters.

Sequencing

Different PrP amino acid sequences have been associated with variation in lesion profiles, incubation periods, and susceptibility to TSE disease in humans and in sheep, cervid, and mouse models. We report the PrP gene sequences for Siberian, Djungarian, and Turkish hamsters. To determine how amino acid sequence might associate with the TSE characteristics we observed here, we compared our new sequences with the published PrP gene ORF of Syrian (*29*), Chinese, and Armenian hamsters (*30,31*).

When comparing the new *prnp* sequences to the Syrian *prnp* sequence, we found considerable variation in nucleotides. Siberian hamsters had 40 nt changes, and Djungarian hamsters had 41. These nucleotide changes resulted in 10 aa substitutions for both species; only 1 aa acid substitution, I215V, distinguished sequences between Siberian from Djungarian hamsters (Figure 7). Only Turkish and Syrian hamsters had 3 nt differences, resulting in 2 aa substitutions. An amino acid Y-to-F substitution was found at codon 6 in the signal sequence, and a heterozygous base
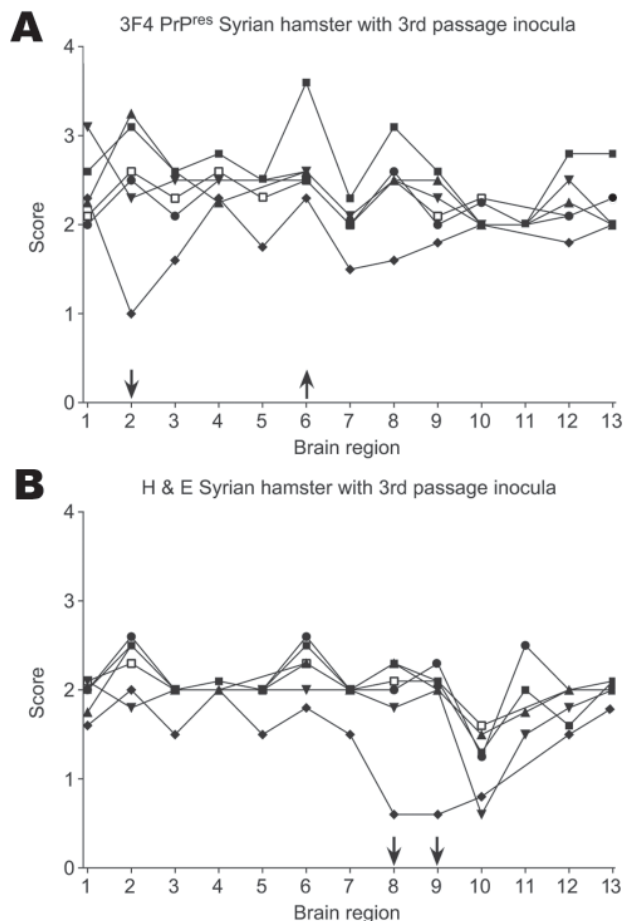

Figure 6. A) Proteinase K–resistant prion protein (PrPres) pathogenicity profiles in Syrian hamsters inoculated with third-passage PrPres. B) Hematoxylin and eosin (H&E)–stained lesion profiles of Syrian hamsters inoculated with brain homogenate derived from third-passage hamsters. Each point represents the average from 6 different animals scored in the following areas: 1, cerebellum; 2, posterior colliculus; 3, superior colliculus; 4, brain stem; 5, spinal cord; 6, thalamus; 7, hypothalamus; 8, hippocampus; 9, cortex; 10, olfactory bulbs; 11, caudate putamen; 12, septal nucleus; 13, tegmentum. Syrian hamster inoculated with third-passage brain homogenate from the following hamster species: ●, Turkish; ■, Djungarian; ♦, Chinese; ▼, Armenian; ▲, Siberian; □, Syrian. Arrows represent differences in multiple regions indicating increases or decreases in vacuolation or PrPres deposition.

was found at codon 103 (encoding both S and N). In addition, Turkish hamsters have a deletion of 1 octapeptide repeat (Δ81–87) on both alleles, a characteristic shared with African Green monkeys (*32*). In humans, deletion of 1 octapeptide repeat occurs at a frequency of 0.5% and is not associated with disease (*33,34*). We found 34 nt changes for Armenian and 37 for Chinese hamsters, resulting in 7 aa substitutions for each hamster species, a finding that agrees with published data (*30*). The 3 aa differences that distinguish Armenian from Chinese hamsters are located at aa positions 103, 108, and 112 (*30*).

Discussion

Of the 6 hamster species inoculated with a well-defined hamster scrapie source, each species not only was susceptible to the agent but also developed unique PrPres biochemical and pathologic characteristics. These characteristics, which included incubation periods, PrPres glycoform patterns, and PrPres distribution in brain, appeared to segregate into related genera with the exception of the genus *Cricetulus* (Armenian and Chinese hamsters). Because TSE characteristics were unique for 2 of 3 genera, a role for host factor involvement is implicated. Our data suggest that PrP amino acid sequence is the host factor responsible for the unique characteristics.

Data from our study and another experimental study (*30*) suggest that not only is the host responsible for determining TSE characteristics such as incubation periods but so is the agent. Our data show that closely related hamsters infected with the same strain of TSE have different incubation periods. These differences cannot be explained by differential expression levels of PrPsen because by Western blot, PrPsen expression was equivalent (Figure 4, panel A). Also, we believe that changes in incubation periods cannot be attributed to differential infectivity titers because only minor variations in titers were found. A second set of experiments showed that the same hamster host inoculated with different strains of TSE had different incubation periods, such as hyper (65 ± 1) and drowsy (168 ± 2) (*23*).

Because incubation periods and altered PrPres deposition in the brain have been linked to polymorphisms in the PrP amino acid sequence (*8,35*), we investigated the possibility that PrPres biochemical and pathologic changes correlate with different host PrP amino acid sequences. When comparing the PrP gene ORF for each of the 6 hamster species, we found 13 possible amino acid substitutions localized to 2 regions, either the N or the C terminus. Hamsters from 2 genera in this study (*Phodopus* and *Mesocricetus*) maintained sequence homogeneity at these 13 residues and had very similar TSE characteristics. Hamsters of the third genus, *Cricetulus,* differed in PrP gene sequence at 3 amino acids within the N-terminal polymorphic region. These polymorphic changes could explain why Armenian and Chinese hamsters have such different incubation periods, PrPres glycoform patterns, and immunohistochemical profiles from each other as well as from the 2 genera *Cricetulus* and *Phodopus.* It is also possible that closely related hamsters have unidentified genes that also influence TSE characteristics.

Our results are consistent with the published data indicating that amino acids 102–139 may control scrapie incubation periods in experimental models (*7,30,36*). In addition, our data suggest that PrPres glycoform profiles and lesion profiles are also associated with this region. Armenian and Chinese hamsters, both in the genus *Cricetu-*

lus, show significant differences from each other, having only 3 aa substitutions at residues 103, 108, and 112 in this N-terminal polymorphic region. In contrast, amino acid sequences from Djungarian and Siberian hamsters were the same in this region, and those from Turkish hamsters matched those from the Syrian hamsters. These findings suggest a direct role for these residues and the N-terminus in determining TSE characteristics. In addition, all 3 genera differ from each other at residue 139, the same position thought to participate in the hydrophobic core potentially stabilizing PrPsen in mice (*37*). The unstructured nature of this region has led some investigators to consider this stretch of residues as a potential nucleation site for conver-
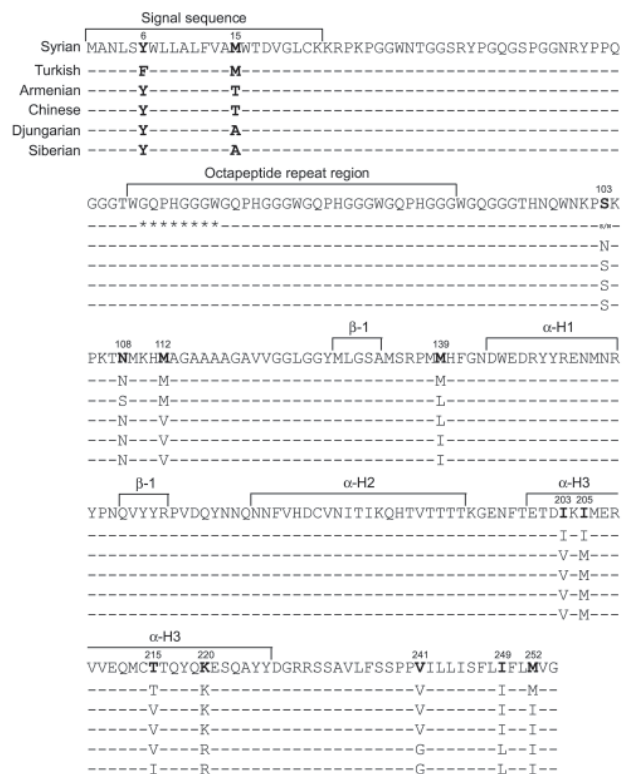

Figure 7. Sequences of prion protein for 3 hamster species. Hamster genomic DNA was purified from whole uninfected hamster brain tissue by using the QIAamp DNA blood maxi 10 kit columns and the solutions and tissue protocol from QIAGEN Dneasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA). PCR products were amplified by using both PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ, USA) and Expand High-Fidelity Taq polymerase (Roche Diagnostic Corp, Indianapolis, IN, USA). Successful amplicons were purified by using QIAquick PCR Purification kit (QIAGEN) according to manufacturer's recommendations and sequenced by using their respective forward and reverse PCR primers with an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence data were stored in the FINCH data management system (Geospiza, Seattle, WA, USA). Assembly and comparisons were made against the Syrian prion protein gene sequence by using Sequencher (Gene Codes, Ann Arbor, MI, USA). Amino acid substitutions are indicated; otherwise, sequence matches that of Syrian hamster.

sion of PrPsen to PrPres (37). Sequence differences in this position may dramatically affect initial conversion events.

Certain amino acid substitutions may play only a minor role in TSE strain profiles. For instance, residue V215T is thought to be responsible for the structural differences between mouse and hamster PrPsen by introducing a bend in helix 3 (38). From our data, T215V/I substitutions may have had a minor in vivo effect with respect to TSE characteristics. Djungarian and Siberian hamsters differed only at this amino acid and had similar glycoform and IHC patterns as well as incubation periods.

Amino acid substitutions identified in this study segregated into genera with similar incubation periods, glycoform profiles, and brain pathologic changes. Because we used only 1 TSE agent, these changes can be attributed to the host factors and not to the agent. A previous study of 3 hamster species also concluded that host rather than agent had a predominant role in determining biochemical and molecular PrPres attributes (30). Because host factors seem to play a larger role in determining the TSE profile, identifying the source of infection in cross-species infections may be difficult, which would be especially worrisome when species important to humans (e.g., sheep, cattle, cervids) are involved. These ruminant species are heterogeneous and not nearly as closely related as the hamsters studied here.

Our study links PrP amino acid sequences to biochemical and pathologic profiles of PrPres in multiple hamster species infected with 263K hamster scrapie. To further investigate how specific amino acid substitutions in PrP are associated with specific TSE characteristics, these hamster and similar PrP gene sequences could be expressed in tissue culture assays and protein modeling experiments. The results of these experiments could broaden our understanding of TSE profiles and the role that PrP amino acid residues play, possibly leading to assays that could identify instances of cross-species transmission.

Acknowledgments

Ms Meade-White is a senior research assistant in the Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases. Her primary interest is infectious diseases of the central nervous system, including transmissible spongiform encephalopathies. Her background includes research in HIV and cyclic neutropenia.

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Prospective Surveillance of Invasive Group A Streptococcal Disease, Fiji, 2005–2007

Andrew C. Steer, Adam Jenney, Joseph Kado, Michael F. Good, Michael Batzloff, Lepani Waqatakirewa, E. Kim Mullholland, and Jonathan R. Carapetis

We undertook a prospective active surveillance study of invasive group A streptococcal (GAS) disease in Fiji over a 23-month period, 2005–2007. We identified 64 cases of invasive GAS disease, which represents an average annualized all-ages incidence of 9.9 cases/100,000 population per year (95% confidence interval [CI] 7.6–12.6). Rates were highest in those >65 years of age and in those <5 years, particularly in infants, for whom the incidence was 44.9/100,000 (95% CI 18.1–92.5). The case-fatality rate was 32% and was associated with increasing age and underlying coexisting disease, including diabetes and renal disease. Fifty-five of the GAS isolates underwent *emm* sequence typing; the types were highly diverse, with 38 different *emm* subtypes and no particular dominant type. Our data support the view that invasive GAS disease is common in developing countries and deserves increased public health attention.

Invasive disease caused by group A streptococci (GAS) occurs when the bacteria infect a normally sterile site. Invasive GAS disease is often life threatening; mortality rate is ≈10%–15% in industrialized countries, increasing to up to 50% in the presence of streptococcal toxic shock syndrome (*1,2*).

A review of the global effects of invasive GAS disease in 2005 estimated that at least 663,000 new cases

and 163,000 deaths occur each year (*3*). Although >95% of these cases and deaths occur in developing countries, few data exist about the epidemiology of these infections in developing countries. In addition, few data describe the clinical signs and symptoms, case-fatality rate, and risk factors associated with invasive GAS disease or the molecular epidemiology of invasive GAS disease in developing countries because most published reports originate from industrialized countries (*4–8*).

We recently reported incidence of invasive GAS infection in Fiji from a retrospective study in the years 2000–2005 (*9*). This study indicated that potentially substantial effects of invasive GAS disease occur in Fiji. We therefore designed a prospective study with active surveillance to ensure good case ascertainment and the acquisition of more detailed clinical information.

Methods

This prospective study was performed at the Colonial War Memorial Hospital in Suva, Fiji, during the 23-month period from December 5, 2005, through November 5, 2007. During the first year of the study, we noticed that a considerable number of invasive group C streptococcal (GCS) and group G streptococcal (GGS) infections occurred. We therefore amended the protocol midway through the study (December 5, 2006) to include cases of invasive GCS and GGS infection (year 2). We analyzed 2 datasets: the first was the data available for 23 months of invasive GAS infections, and the second was the data available in year 2 for the 11 months of surveillance of all invasive β-hemolytic streptococcal infections (GAS, GCS, and GGS).

Setting

Fiji is a nation of ≈330 islands located in the Western Pacific. It has a population of 827,900 persons consist-

ing of 2 major racial groups, indigenous Fijians (57.3%) and Indo-Fijians (37.6%) (*10*). Approximately 49% of the population lives in rural areas (*10*). Of 177 nations on the United Nations Development Programme Human Development Index, Fiji is number 90. Fiji has a gross domestic product per capita of US$6,066, and indexes of wealth are similar for the 2 major racial groups; Indo-Fijians are at a slight disadvantage. The 2002–2003 Household Income and Expenditure Survey estimated that 35.6% of the Indo-Fijian population live in poverty compared with 34.2% of the indigenous Fijian population (*11,12*). Life expectancy at birth is 63.8 years for men and 66.8 years for women and is similar for both major racial groups (*11,12*). Fiji has an infant mortality rate of 17.2/1,000 population, slightly higher for Indo-Fijians at 19.1/1,000 than for indigenous Fijians at 16.8/1,000 (*11,12*). The major hospital, the Colonial War Memorial Hospital (CWMH), is located in the capital, Suva, on the main island of Viti Levu, and primarily serves the Central Division, the largest of the 4 administrative divisions in Fiji. Five subdivisional hospitals are located in the Central Division of Fiji, but most seriously ill patients are admitted to CWMH. This circumstance is due to the 568-bed capacity of CWMH compared with the 81-bed capacity of all 5 subdivisional hospitals combined. CWMH has ≈20,500 admissions per year; the number of indigenous Fijians and Indo-Fijians admitted is proportionate to the population of the Central Division. The total population of the Central Division in 2007 was 340,843: 65% indigenous Fijians and 30.2% Indo-Fijians (*10*). The remainder of the population includes a substantial Chinese population as well as Europeans and Pacific Islanders, including persons from Rotuma, a volcanic island >400 km north of Fiji (it is politically part of Fiji, but its people are ethnically distinct from indigenous Fijians) (*13*).

Surveillance and Case Definitions

Admission registers at CWMH were checked, and treating physicians were consulted daily for any admitted patient that may have had invasive GAS disease, including those admitted with necrotizing fasciitis, sepsis, and soft tissue infections. The diagnostic microbiology laboratory at CWMH was also contacted daily for any new β-hemolytic streptococcal isolates obtained from sterile and nonsterile sites. We used a consensus case definition of invasive GAS disease developed in 2005 by a World Health Organization/US National Institutes of Health working group; the definition is currently being prepared for publication (F. Rubin, pers. comm.) (Table 1). Cases of streptococcal toxic shock syndrome were defined according to published criteria (*14*). Clinical data were collected from review of the medical records and from information provided by the treating physicians when necessary.

Laboratory Methods

All blood cultures collected at CWMH were processed in an automated blood culture machine in the diagnostic microbiology laboratory. Isolates that were suspected of being GAS, GCS, or GGS were subcultured onto sheep blood agar and regrown for Lancefield grouping (Oxoid; Cambridge, UK). Susceptibility testing was performed on sheep blood Mueller-Hinton agar by the disk-diffusion method by using Clnical Laboratory Standards Institute guidelines against a panel of 4 antimicrobial drugs: penicillin (10 μg), erythromycin (15 μg), clindamycin (2 μg), and chloramphenicol (30 μg) (Oxoid). Isolates were transported to the Queensland Institute of Medical Research for *emm* sequence typing according to the standard methods developed by the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) (*15*).

Statistical Calculations

We used the population of the Central Division from the 2007 national census as the basis for denominator calculations. Because surveillance occurred during 2005 (1 month), 2006 (12 months), and 2007 (10 months), we extrapolated a total population figure for 2005 and 2006 using the pro rata difference between the last official census in 1996 and the latest census in 2007. In calculating average annualized rates, the denominator of person-months was calculated by multiplying each year's population by

Table 1. Case definitions for invasive GAS disease, Fiji, 2005–2007*	
Disease	Case definition
Definite	Either of the following:
	1. The isolation of GAS from a normally sterile site (e.g., blood, cerebrospinal fluid, or other sterile fluid/tissue).
	2. Clinical presentation of necrotizing fasciitis with evidence of GAS infection (e.g., the presence of typical gram-positive cocci on Gram stain or positive streptococcal serology).
Probable	Any of the following:
	1. A classic presentation of necrotizing fasciitis without microbiological confirmation.
	2. Cellulitis in a patient who is moderately or severely unwell (i.e., unwell and history of parenteral antibiotics and/or admission to hospital) and microbiological confirmation (i.e., group A streptococcal culture of swab or positive streptococcal serology).
	3. Other clinically significant infection in a patient who is moderately or severely unwell (i.e., unwell and history of parenteral antibiotics and/or admission to hospital), in conjunction with positive group A streptococcal culture from deep wound swab or biopsy from surgical infection site.

*GAS, group A streptococci.

the number of months of surveillance for each year (i.e., 2005 population by 1 month, 2006 by 12 months, and 2007 population by 10 months) and adding these totals. The total number of cases over the 23 months was then divided by the total person-months and multiplied by 12 to give average annualized incidence rates with binomial exact 95% confidence intervals (CI). Incidence rate ratios were used to compare rates between ethnic groups. We used χ^2 calculations of odds ratios (ORs) for univariate analysis of categorical data. Data were analyzed by using Stata version 10.0 (StataCorp, College Station, TX, USA).

Ethical Approval

Ethical approval was obtained from the Fiji National Research Ethics Review Committee, the Fiji National Health Research Committee, the University of Melbourne Human Research Ethics Committee, and the Queensland Institute of Medical Research Human Research Ethics Committee. We asked all patients for their consent for the collection of more detailed clinical and outcome data as well as for the transport and testing of the clinical isolates. Information sheets in Fijian and English were provided to potential participants before enrollment. We required that all patients provide written informed consent before information was collected. Children were only enrolled if a parent or guardian provided written consent, and we also required written assent from children ≥10 years of age. When patients did not consent, the case was noted for incidence calculations, but more detailed clinical and outcome data were not collected.

Results

Invasive Group A Streptococcal Infections

Epidemiologic Data

Sixty-four cases of invasive GAS disease occurred during the 23 months of surveillance. Sixty-two cases met the criteria for a definite case, and 2 cases met the criteria for a probable case. The average annualized all-ages incidence of invasive GAS disease (both definite and probable) in the Central Division was 9.9 cases/100,000 population/year (95% CI 7.6–2.6). There were an equal number of male and female case-patients. The median age of patients with invasive GAS disease was 51.6 years (interquartile range [IQR] 27.6–66.4 years). The youngest patient was aged 1 month, and 7 patients were <1 year of age, representing an incidence of 44.9/100,000 population (95% CI 18.1–92.5) in this age group (Figure). The peak incidence occurred in patients ≥65 years (incidence 80.6/100,000, 95% CI 46.1–130.8). Fifty-three cases occurred in indigenous Fijians (incidence 13.1/100,000, 95% CI 9.8–17.1), 5 cases in Indo-Fijians (incidence 2.5/100,000, 95% CI 0.8–5.9), and 6 cases in persons
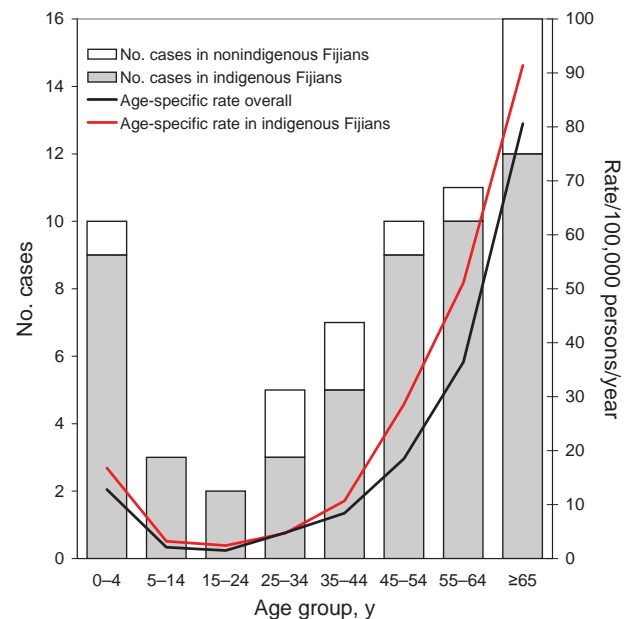

Figure. Invasive group A streptococcal disease in the Central Division of Fiji, December 5, 2005–November 5, 2007.

of other races (incidence 12.8/100,000, 95% CI 4.7–27.8). Of the 6 cases in persons of other races, 4 patients were Rotuman (incidence 47.4/100,000, 95% CI 12.9–121.4). When adjusted for the population of the Central Division, the incidence rate ratio for invasive GAS disease in indigenous Fijians versus other races was 2.9 (95% CI 1.5–6.1).

Clinical Data

Of the 64 case-patients with invasive GAS disease, informed consent to collect clinical and outcome information was obtained from 60. Soft tissue infection (with associated bacteremia) was the most common clinical infection (23 cases [38%]; Table 2); followed by bacteremia with no clinical focus of infection (17 cases [28%]), and necrotizing fasciitis (4 cases [7%]). Three cases (5%) fulfilled the criteria for streptococcal toxic shock syndrome; the clinical focus for these infections was soft tissue infection in 2 case-patients and pneumonia in the third case-patient. Group A streptococci were isolated from blood cultures of 55 (92%) of the 60 case-patients, from both blood culture and other sterile fluid of 4 case-patients, from sterile fluid alone of 3 case-patients, and from nonsterile sites (breast abscess fluid and leg cellulitis swab) of the 2 patients with probable cases. GAS was isolated from blood culture for all 4 case-patients from whom consent to obtain further clinical information was not obtained.

Thirty-eight case-patients (63%) had at least 1 known coexisting chronic medical condition (Table 3). Diabetes was the most common coexisting condition (25 patients [42%]),

Table 2. Clinical signs and symptoms in patients with invasive GAS infection, by age, Fiji, 2005–2007*

Clinical signs/symptoms	No. patients by age group			Total no. (%) patients
	0–14 y	15–49 y	>50 y	
Soft tissue infection	2	9	12	23 (38)
Bacteremia with no clinical focus	5	5	7	17 (28)
Septic arthritis	2	1	5	8 (13)
Necrotizing fasciitis	–	2	2	4 (7)
Pneumonia	2	–	2	4 (6)
Gynecologic infection	–	1	1	2 (3)
Osteomyelitis	1	–	–	1 (2)
Peritonitis	–	–	1	1 (2)
Total no. (%) patients	12 (20)	18 (30)	30 (50)	60
*GAS, group A streptococci.				

and was found in 7 of the 12 patients with soft tissue infection who were >50 years of age. No significant difference was found in the rates of underlying disease for indigenous Fijians (62%) compared with rates for persons of other races (70%; p = 0.6). Eighteen patients (30%) had current or recent impetigo. Impetigo was more common in younger patients and was present in 7 of the 10 patients with invasive GAS disease who were <5 years of age and in 4 of the 7 patients <1 year of age. No cases of varicella-zoster virus infection were found in this study, and only 1 patient reported the recent use of a nonsteroidal antiinflammatory drug.

Outcome and Case-Fatality Rate Data

Of the 60 case-patients for whom outcome data were available, 19 died, representing a case-fatality rate of 32%. The case-fatality rate increased with patient age; peak fatality rate was 44% in those >65 years of age. The odds of death for patients ≥50 years compared with those of the rest of the patient group was 3.1 (95% CI 0.9–11.7). The youngest patient to die from invasive GAS disease was 1 year and 2 months of age; this was the only death in patients <25 years. No significant difference was found in case-fatality rate for indigenous Fijians (32%) compared with that for persons of other races (30%; p = 0.9). All deaths occurred within 6 days of admission; 5 deaths (26%) occurred within the first 24 hours, 12 deaths (63%) within the first 48 hours, and 15 deaths (79%) within the first 72 hours. The clinical sites of infection in the group of patients that died were similar to the pattern seen overall: 7 patients had a soft tissue infection, 9 patients had bacteremia alone, 2 patients had pneumonia, and 1 had necrotizing fasciitis. All 3 patients with streptococcal toxic shock syndrome died. A coexisting chronic medical condition was associated with an increased risk for death from invasive GAS disease (OR 4.6, 95% CI 1.0–27.7). Twelve patients with diabetes died (OR 3.7, 95% CI 1–13.7), and 8 patients with renal disease died (OR 5.2, 95% CI 1.2–24.2).

Fourteen patients (23%) required surgery as part of their treatment regimen, and 2 of these patients had a leg amputated below the knee. Five patients (8%) were admitted to the intensive care unit, and 1 patient required mechanical ventilation. Twenty-nine patients (48%) were admitted to the hospital for >10 days. Of the 41 survivors of invasive GAS infection, 16 had a residual disability (39%). Overall, 35 patients (58%) died or had a disability as a result of an invasive GAS infection.

Laboratory Data

Susceptibility testing was performed on 57 of the 60 isolates, and all 57 isolates were susceptible to all 4 antimicrobial drugs tested. Fifty-five of the 60 isolates were able to be *emm* sequence typed (Table 4); 37 different *emm* types and 38 different *emm* subtypes with no particular dominant types emerged from this series. The most common subtypes were *emm100* and *emm76.4* (4 cases each), followed by *emm11*, *emm33*, and *emm106* (3 cases each). No *emm* type 1 isolates were found. Eight of the 55 isolates *emm* typed were in nonindigenous Fijian patients, and of these, only 1 *emm* subtype was also observed among indigenous Fijian patients.

Invasive β-hemolytic Streptococcal Infections after December 5, 2006

Epidemiologic Data

In addition to the GAS infections, 4 cases of invasive GCS infection and 14 cases of invasive GGS infection

Table 3. Underlying coexisting medical conditions in patients with invasive GAS disease, Fiji, 2005–2007*

Condition	No. (%) patients
Diabetes	25 (42)
Renal disease	13 (22)
Cardiac disease	13 (22)
Malignancy	5 (8)
Immunosuppression	2 (3)
Lung disease	3 (5)
Liver disease	1 (2)
Any coexisting condition	38 (63)
Only 1 coexisting condition	21 (35)
2 coexisting conditions†	11 (18)
>2 coexisting condition†	6 (10)
*GAS, group A streptococci.	
†Nine patients had diabetes and renal disease.	

were found in year 2, making a total of 44 cases of invasive β-hemolytic streptococcal infection during the 11 months of surveillance in year 2. This represents an annualized all-ages incidence of 14.1 cases/100,000 population/year (95% CI 10.2–18.9), an incidence rate of invasive GCS disease of 1.3/100,000 (95% CI 0.3–3.3), and an incidence rate of invasive GGS disease of 4.5/100,000 (95% CI 2.5–7.5). Overall, 24 male patients and 20 female patients were infected. The peak incidence was in those >65 years of age (incidence 104.7/100,000, 95% CI 50.2–192.4), and 3 cases occurred in infants. Thirty-four cases occurred in indigenous Fijians (incidence 17.4/100,000, 95% CI 12–24.3), and 10 cases occurred in persons of other races (incidence 8.6/100,000, 95% CI 4.1–15.8); incidence rate ratio for indigenous Fijians was 2 (95% CI 1–4.6).

Clinical, Outcome, and Laboratory Data Relating to GCS and GGS Disease

Of the 18 cases of invasive GCS and GGS disease, informed consent to collect clinical and outcome information was obtained for 12 of 14 case-patients with invasive GGS infection and for all 4 case-patients with invasive GCS infection. The clinical spectrum of GCS and GGS infection was similar to that for invasive GAS infection, although the number of patients with bacteremia with no clinical focus was higher, and a case of meningitis in a neonate caused by GCS was added (Table 5). Similar to the experience of patients with invasive GAS disease, a high proportion of patients with invasive GCS and GGS disease had a coexisting chronic medical condition (69%); 44% of patients had cardiac disease, 31% of patients had diabetes, and 6% had renal disease. Six deaths occurred (38%). *emm* sequence typing was performed on 12 isolates (2 GCS and 10 GGS), and 9 different *emm* types were found (Table 4). One was *emm12*, which is normally associated with GAS.

Discussion

This study confirms the extensive effects that invasive GAS infections have in Fiji and adds to the small amount of existing data indicating that the incidence and case-fatality rates of these infections are ≈3–4 times higher in developing countries than in industrialized countries. A study in Kenya found that the incidence of GAS bacteremia in children <15 years of age was 13/100 000 population and that mortality rate was 25% (*16*), similar to the results in our study. In indigenous populations in wealthy countries, such as Aboriginal Australians and Native Americans, the all-ages incidence of invasive GAS disease is as high as 82.5/100,000 and 46/100,000, respectively (*2,17,18*).

We observed a nearly 3-fold higher risk for invasive GAS disease in indigenous Fijians. The reasons are not entirely clear and do not appear to be related to socioeconomic status or to coexisting conditions, including diabetes; detailed studies of diabetes in Fiji have shown that the prevalence of diabetes is higher in Indo-Fijians (*19,20*) than in indigenous Fijians. One possible explanation is that

Table 4. *emm* sequence subtypes of 55 invasive GAS isolates and 12 invasive GCS and GGS isolates from hospital surveillance, Fiji, 2005–2007*

GAS *emm* subtype	No. isolates (n = 55)
emm11†	3
emm14.4†	1
emm15.1	1
emm18.12†	1
emm19.4†	1
emm22†	1
emm33†	3
emm52.1	1
emm53	1
emm57	2
emm58	1
emm60.1	1
emm60.2	1
emm63.3	1
emm69.1	1
emm70	1
emm73	2
emm75.1†	1
emm76.4†	4
emm77†	2
emm81.3	1
emm82.1	1
emm86.2	1
emm87	1
emm100	4
emm101†	1
emm104	1
emm105	1
emm106	3
emm110	2
emm113	1
emm116.1	1
emm123	1
st2037	1
st2147	1
st6030.1	1
st854.1	2
stD631	1
GCS and GGS *emm* subtype	No. isolates (n = 12)
emm12.8†	1
stC36	2
stC922	1
stG245	2
stG643	1
stG652	1
stG840	1
stG5420	1
stc74a	2

*GAS, group A streptococci; GCS, group C streptococci; GGS, group G streptococci.
†*emm* subtypes included in the 26-valent vaccine.

Table 5. Clinical signs and symptoms of invasive GCS and GGS infection, Fiji, 2005–2007*

Clinical signs/symptoms	GCS infection	GGS infection	Total
Bacteremia without clinical focus	–	7	7
Soft tissue infection	1	3	4
Endocarditis	1	1	2
Arthritis	–	1	1
Pneumonia	1	–	1
Meningitis	1	–	1
Total	4	12	16
*GCS, group C streptococci; GGS, group G streptococci.			

GAS skin disease is more extensive in indigenous Fijian communities and that this leads to a higher risk for invasive GAS disease. We have observed a higher prevalence in Fiji of bacterial skin disease in indigenous Fijian children (A. Steer, unpub. data), and high rates of GAS skin disease have been found in Pacific Islanders in other parts of the world, including in Maoris and other Polynesians in New Zealand (21,22).

In most industrialized countries, the annual incidence of invasive GAS disease is 2.5–3.5/100,000 population (8,23), and the case-fatality rate is 7%–15% (6,8,23–26). Although some epidemiologic similarities were found between invasive GAS disease in these countries and in Fiji, such as the high rates for elderly persons with coexisting medical conditions, as well as in the clinical spectrum of disease, major differences stand out. Not only was the all-ages incidence rate higher, but we also found that the incidence rate for children in Fiji was disproportionately high when compared with rates in industrialized countries. For example, the average annualized incidence rate for infants in the United States in 2000–2004 was 5.3/100,000, whereas in Fiji it was 44.9/100,000 (8).

The high case-fatality rate in our study (32%) may reflect the late appearance of clinical signs and symptoms, particularly because such a high proportion of deaths occurred in the first 24–48 hours after admission. It may also reflect inadequate treatment of invasive GAS infection, particularly of early sepsis, possibly because of reduced access to intensive care facilities and advanced therapies. The rate of diabetes was also higher in our study than in other studies, potentially further increasing the case-fatality rate.

The *emm* typing profile of invasive GAS isolates was different than that in industrialized countries. In most epidemiologic studies in wealthy countries, a high proportion of disease is caused by a small number of *emm* types that include *emm1* (22% of *emm* types in the United States), *emm3* (9%), and *emm28* (9%) (8). None of these *emm* types were found in our study, and we observed a lack of dominant *emm* types. The diversity in *emm* types may reflect the high diversity of GAS in impetigo lesions, as can be seen in tropical settings other than Fiji (27).

We observed a substantial number of non-GAS invasive cases in the second year of the study after we expanded our surveillance to include these cases. Individual cases of GCS and GGS invasive disease have been described before (28,29), but prospective surveillance has not been performed. Our data suggest that surveillance of invasive GAS disease in developing countries should also include surveillance of invasive GCS and GGS disease because the disease profile of these organisms is similar and because control strategies, including vaccines that can offer cross-protection between Lancefield groups, may also be similar.

Identification of factors associated with invasive GAS disease, in particular deaths from invasive GAS disease, can guide disease prevention and disease management efforts. In adults, diabetes was an important risk factor that was associated with death. In children, impetigo appeared to be associated with invasive GAS disease. Control of diabetes in the elderly and control of impetigo in the young may be important disease prevention public health goals. With so many deaths occurring shortly after hospital admission, improved recognition and case management of early sepsis may be important disease management goals. The findings of this study also have implications for GAS vaccine development. Several GAS antigens have been identified as potential vaccine candidates. However, only 1 GAS vaccine, a 26-valent M protein vaccine, has reached phase I and II clinical trials (30). Serotypes included in this vaccine were chosen if they were common *emm* types in the United States (31,32). Although clinical development of the 26-valent vaccine is in its early stages, our results raise concerns about the applicability of this vaccine to developing country settings, because only 10 of 37 *emm* types in our study are types included in the vaccine, accounting for 33% of GAS isolates.

Our study confirms that invasive disease caused by GAS and other β-hemolytic streptococci has substantial effects in Fiji. This study provides the detailed epidemiologic and clinical data on invasive GAS infections from a developing country.

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dren's Hospital in Melbourne. His work in the Fiji Islands is focused on investigating the epidemiology of group A streptococcal infections as part of a project aimed at development of a novel group A streptococcal vaccine.

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Bacterial Phenotype Variants in Group B Streptococcal Toxic Shock Syndrome[1]

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We conducted genetic and functional analyses of isolates from a patient with group B streptococcal (GBS) necrotizing fasciitis and toxic shock syndrome. Tissue cultures simultaneously showed colonies with high hemolysis (HH) and low hemolysis (LH). Conversely, the HH and LH variants exhibited low capsule (LC) and high capsule (HC) expression, respectively. Molecular analysis demonstrated that the 2 GBS variants were of the same clonal origin. Genetic analysis found a 3-bp deletion in the *covR* gene of the HH/LC variant. Functionally, this isolate was associated with an increased growth rate in vitro and with higher interleukin-8 induction. However, in whole blood, opsonophagocytic and intracellular killing assays, the LH/HC phenotype demonstrated higher resistance to host phagocytic killing. In a murine model, LH/HC resulted in higher levels of bacteremia and increased host mortality rates. These findings demonstrate differences in GBS isolates of the same clonal origin but varying phenotypes.

Group B streptococci (GBS) are a major cause of sepsis in neonates and pregnant women. The incidence of invasive GBS disease in nonpregnant adults is growing, in particular in elderly persons and in those with chronic underlying conditions (e.g., diabetes mellitus) (*1*). Recently, cases of the severe, life-threatening syndromes of necrotizing fasciitis (*1*) and toxic shock syndrome due to GBS have been reported in neonates (*2*) and immunocompromised persons (*3*), reminiscent of a disease course more commonly associated with group A streptococci or *Staphylococcus aureus*. We report a case of GBS necrotizing fasciitis and toxic shock syndrome in a previously healthy person. We discovered 2 specific phenotypic variants of the bacterium from the tissue site of infection. Genetic and functional analysis of these variants provides insight into the potential contribution of specific bacterial virulence factors to these emerging GBS clinical syndromes.

Of GBS virulence factors, 2 of the best characterized are its exopolysaccharide capsule and the surface-associated toxin, β-hemolysin/cytolysin (β-h/c). The capsule contributes to immune resistance by inhibiting complement deposition and activation on the bacterial surface, thereby reducing opsonophagocytic clearance (*4*). GBS production of β-h/c is encoded by the genes of the *cyl* operon (*5,6*) and is associated with direct lysis of a variety of eukaryotic cell types (*7–9*), inflammatory activation (*10–12*), and virulence in animal models (*10,13,14*). GBS β-h/c expression is linked to expression of an orange pigment with antioxidant properties (*6,15*), and these 2 factors act in concert to impair macrophage-based immune clearance (*16*).

In the traditional clinical view of invasive GBS pathogenesis, a bacterial isolate enters a normally sterile site from a focus of mucosal colonization or recent acquisition of the pathogen. However, as the present case will illustrate, selective pressures in vivo may cause differential expression of certain GBS surface components during colonization or dissemination. Thus, in vivo pathogenesis from a bacterial perspective is likely more dynamic.

Case Report

A previously healthy 50-year-old man was admitted to the hospital with fever, severe pain and swelling of the right shoulder and arm, 1 week after moderate trauma. The extremity was erythematous, markedly swollen, and intensely tender. In the emergency department, the man's condition rapidly deteriorated to septic shock. After receiving immediate support with oxygen, intravenous fluids, and antimicrobial agents (penicillin G, 3 g, 4×/day, and a single dose of 120 mg gentamicin), he was transferred to the intensive care unit. In addition to mechanical ventilation and vasopressors, medical treatment included intravenous immunoglobulins and corticosteroids. Because necrotizing fasciitis was suspected, wide debridement was performed, which confirmed the clinical diagnosis. In tissue specimens obtained during the operation, gram-positive cocci with typical streptococcal morphologic features were abundant. After samples underwent overnight culture on blood agar plates and the organisms were identified to species level, GBS was isolated. This pathogen also grew in cultures of blood obtained while the patient was in the emergency room. Antimicrobial drug treatment was changed to the combination of clindamycin and penicillin G. After a total of 3 repeated debridements and antimicrobial drug treatment for 6 weeks, the outcome was favorable. At follow-up after 6 months, the patient had only a slight radiating pain in the arm.

Methods

Sources of Bacteria

We included the following in specific, comparative assays: 2 serotype V GBS strains isolated from a colonized person (VK9) and a neonate with sepsis (CNCTC), GBS NEM316, and 1 group A streptococci serotype M1T1 isolate from a patient with streptococcal toxic shock syndrome (isolate 5448).

CAMP Test, Serotyping, Antimicrobial Susceptibility, and Pulsed-Field Gel Electrophoresis

Identification of the isolates was confirmed by CAMP testing, and serotype determination was achieved by using a coagglutination typing kit (Essum, Bacterium AB, Umeå, Sweden). Antimicrobial susceptibility and MICs were determined by the Kirby-Bauer disk diffusion method and by Etest (AB Biodisk, Solna, Sweden), respectively. GBS isolates were subjected to pulsed-field gel electrophoresis (PFGE) by using the restriction enzymes *Sma*I or *Xma*I (New England Biolabs, Ipswich, MA, USA) or *Apa*I (Promega, Madison, WI, USA), as described (*17*).

Measurement of Pigment and Hemolytic Activity

Pigment was extracted as described (*16*). The optical density (OD) of the pigment extracts was measured at a di-

lution of 1:4 in a spectrophotometer (WPA Biowave, Biochrom, Cambridge, UK). The hemolytic activity was determined by measuring hemoglobin release in the supernatant (by OD), after pigment extracts were incubated with an equal volume of 1% sheep erythrocytes for 1 h. Phosphate-buffered saline (PBS), glucose alone, and erythrocytes lysed with 0.1% sodium dodecyl sulfate (SDS) were used as negative and positive controls, respectively. The results were related to SDS (100%) and expressed as hemolytic capacity. The hemolytic titer was assessed by a microtiter dilution method, as described previously (*7*).

Capsule Expression

Buoyance density of overnight bacterial cultures was determined by Percoll gradient centrifugation as described previously (*18*). To analyze surface sialic acid expression, strains were grown to mid log phase, washed, and resuspended in PBS to an OD of 0.4. Sialic acids were hydrolyzed with mild acid, then filtrated, neutralized, and derivatized as previously described (*19*) for quantitative analysis by high-performance liquid chromatography. To visualize capsule expression, we incubated isolates on blood agar plates and in Todd-Hewitt broth (THB) overnight. Isolates were then washed and fixed with Karnovsky solution. After polymerization, samples were sectioned with an ultramicrotome (Reichert-Jung Ultracut E, Leica, Wetzlar, Germany), and analyzed by transmission electron microscopy (FEI, Philips, Morgani 268D, Aachen, Germany).

Genetic Analysis

PCR was used to amplify the *cylA*, *cylE*, *covR*, *covS*, *rovS,* and *stk1* genes, as described previously (*6*), and sequences of amplicons were compared with the published genome of GBS serotype Ia strain A909 (*20*). Sequence alignment for all the genes showed >99% identity among GBS strains of different serotypes.

For heterologous expression of *covR/S*, the region was amplified by PCR from the GBS genome by using the forward primer 5′-GCGTCTAGAGAATAAGAAGGTTGG TGTAGATGGG-3′ and reverse primer 5′-CGCGGATTC GAAGCGCCTCTCTTATCACCTC-3′. The 2,286-bp amplicon was captured in pTTOPO according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), then subcloned into expression vector pDCerm (*21*). The resulting pDC-CovRS plasmid was introduced into GBS by electroporation (*22*). Transformants were identified by erythromycin resistance, and plasmid presence was confirmed by PCR.

RNA Isolation and Reverse Transcription–PCR

Total bacterial RNA was extracted from overnight cultures of GBS by using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) per manufacturer's instructions, except

that bacteria were mechanically disrupted by using tubes with glass beads (Lysing Matrix B, MP Biomedicals, Solon, OH, USA). RNA samples were DNase treated (Turbo DNA-free; Ambion, Austin, TX, USA) to remove any contaminating DNA. One microgram of RNA was reverse transcribed to cDNA (Superscript First-Strand Synthesis Kit; Invitrogen) and used for PCR amplification with the following primer sets: *cfb* forward 5′-CTGGAACTCTAGTGGCTGGTG-3′ and *cfb* reverse 5′-CCATTTGCTGGGCTTGATT-3′; *cylK* forward 5′-ATTTATCTGGCGATCGGTTG-3′ and *cylK* reverse 5′-CCTTTGGCAAACCAATTAAATAAC-3′; *cylE* forward 5′-GTCGTA GTGGACAGGCAATCAC-3′ and *cylE* reverse 5′-CGAAATGATCGACAATGCAG-3′; *cpsG* forward 5′-CATGAACAGCAGTTCAACCG-3′ and *cpsG* reverse 5′-CTGACATAAACGTCGCTGGAC-3′; and *gyrA* forward 5′-CTTGGTGATGGGACGTTCAGG-3′ and *gyrA* reverse 5′-GCTGAAGCAGCACGACGAAC-3′. PCR mixtures contained primers at a concentration 1 μM and PCR mix (Supermix; Invitrogen) in a volume of 15 μL. Samples that had been prepared without reverse transcriptase served as controls for DNA contamination. The PCR products were visualized by electrophoresis on a 1% agarose gel containing ethidium bromide.

Measurement of Growth Dynamics and Phenotype Stability

Bacterial growth rates were determined in THB and THB plus 1.5% yeast extract (THB + Y) by OD_{600} determination and enumeration of CFUs. Measurements were performed in triplicate. To evaluate phenotype stability, we passaged each variant isolate on blood agar (7 passages) and in various media (3 passages), including THB, THB + Y, Granada, and chromogenic (Strepto B ID agar; bioMérieux SA, Marcy-l'Etoile, France).

Cytokine Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of 5 healthy donors by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Axis Shield PoC AS, Oslo, Norway). PBMCs were stimulated with live bacteria (multiplicity of infection [MOI] ≈1:1) for 2 h. Uninfected PBMCs served as negative controls. Interleukin (IL)-1β, IL-8, and tumor necrosis factor α (TNF-α) in cell culture supernatants were determined by Luminex multiplex assays (BioSource International, Camarillo, CA, USA) and the Luminex[100] instrument (Luminex, Austin, TX, USA).

Murine Model of GBS Infection

Male CD-1 mice (Charles River Laboratories, San Diego CA, USA) 6–8 weeks of age were injected intraperitoneally with $6–8 \times 10^6$ (low inoculum) or $5–7 \times 10^7$ CFU (high inoculum) of either GBS phenotype variant and monitored for survival. After 6 h, blood was collected by retro-orbital puncture and assessed for levels of bacteremia by serial dilution plating on blood agar plates. Ethics approval for animal experimentation was obtained from the Animal Care Program of the University of California, San Diego, CA, USA.

Determinations of Bacterial Growth and Killing Rates in Human Whole Blood

Bacterial growth rates in freshly collected whole blood from 3 nonimmune human donors were determined by enumeration of CFUs. The blood was incubated with 10% volume of an overnight bacterial culture under mild agitation. Measurements were performed in duplicate.

For whole blood killing assays, inocula of 100 CFU in 100 μL were mixed with 300 μL human blood (n = 5 donors) in heparinized tubes and incubated for 1–3 h with mild agitation. Dilutions were plated on blood agar for enumeration of CFU. Autologous plasma from nonimmune donors were used as controls.

Neutrophil Opsonophagocytic Killing Assays

Bacteria (CFUs ≈10^6) grown as described above were incubated with 10% normal human serum (i.e., source of complement) for 10 min and then mixed with autologous neutrophils (MOI ≈1:1) from the 5 nonimmune donors. Neutrophils were isolated by density gradient centrifugation using Polymorphprep solution (Axis Shield PoC AS). Controls included samples containing heat-inactivated serum and neutrophils, serum without neutrophils, and PBS. Immediately before and after 60 min incubation, 100 μL of sample solutions were removed and plated on blood agar plates (*23*). To determine the kinetics of GBS survival within neutrophils, we pelleted and resuspended the cells in minimum essential medium supplemented with L-glutamine, 125 μg/mL gentamicin (GIBCO, Invitrogen) and 5 μg/mL penicillin G (*24*). After 30, 60, and 90 min, cells were lysed and CFU enumerated. Samples with only bacteria were used as a control for the bactericidal effects of antimicrobial drugs.

Statistical Analysis

Groups were compared by using a nonparametric paired test (Wilcoxon signed-rank test) and Kaplan-Meier plot; p values <0.05 were considered significant. Statistical calculations were performed by using GraphPad Prism, Version 4.03 (Graph Software, San Diego, CA, USA).

Results

Pigment and Hemolytic Activity

Culture of a tissue sample on blood agar plates displayed GBS colonies with 2 different phenotypes, either high hemolytic (HH) or low hemolytic (LH) (Figure 1, pan-
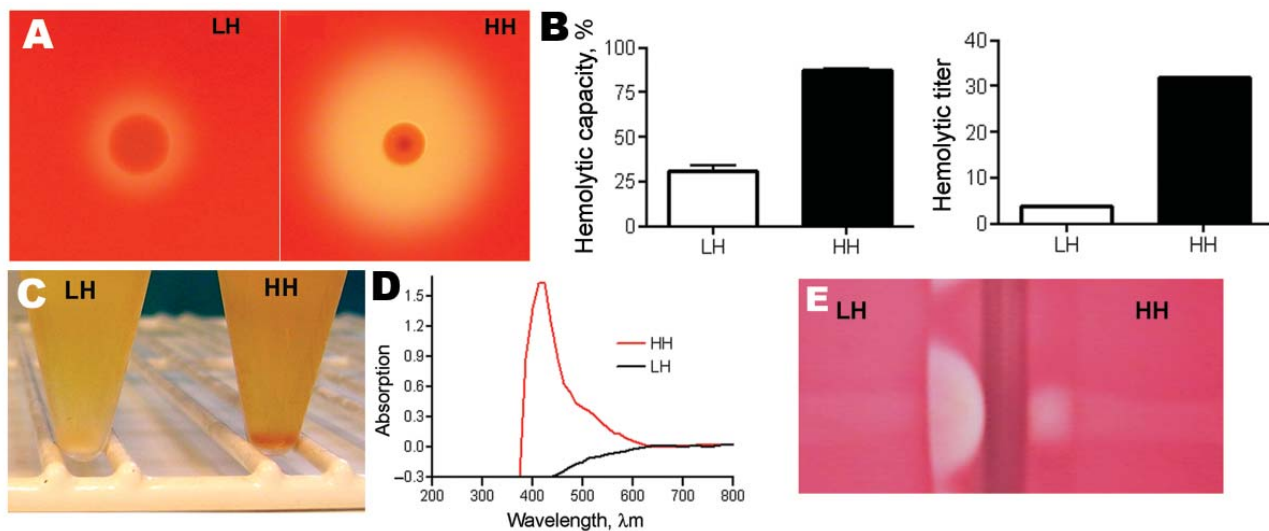
Figure 1. A) Hemolytic zone on blood agar plate after 48 h: Low hemolytic (LH) colony and high hemolytic (HH) colony. B) Hemolytic activity of the pigment extract presented as hemolytic capacity (left graph) relative to that of sodium dodecyl sulfate (100%) and as hemolytic titer (right graph) evaluated with a microdilution assay. Error bars indicate SEM. C) Phenotypic appearance of group B streptococci after overnight culture in Todd-Hewitt broth plus 1.5% yeast extract, displaying a white pellet (LH) and an orange pellet (HH). D) Absorbance profile of the pigment extract. E) Results of CAMP testing, which display a stronger reaction with the LH than with the HH phenotype.

el A). The difference in hemolysis was corroborated by 2 different assays, which showed a 4- to 8-fold difference in hemolytic potential between the HH and LH variants (Figure 1, panel B). The HH phenotype had strong orange pigmentation; the pigmentation of the LH variant was difficult to detect in culture pellets or by spectrophotometric analysis (Figure 1, panels C and D). In CAMP testing, however, the HH phenotype displayed a weaker reaction than the LH variant (Figure 1, panel E). The distinct phenotypes of the 2 isolates proved to be stable after up to 7 passages in various media.

Encapsulation

Evaluation of encapsulation by buoyant density centrifugation showed high density for the HH variant (consistent with low encapsulation [LC]) and low density for the LH strain (consistent with high encapsulation [HC]) (Figure 2, panel A). This difference in encapsulation was further confirmed by direct quantification of sialic acid levels (Figure 2, panel B) and by imaging with transmission electron microscopy (Figure 2, panel C).

Clonal Origin of HH/LC and LH/HC Phenotypic Variants

Both phenotypes belonged to capsular serotype Ib and showed equivalent patterns of antimicrobial drug susceptibility or resistance. PFGE showed identical banding patterns after 3 restriction enzymes were used, which indicates that the 2 phenotypes had the same clonal origin. To assess whether a genetic mutation could explain the observed phenotypic difference, we sequenced genes implicated in β-h/c production or regulation. Although ampli-

fication and sequencing of *cylE*, *cylA*, *covS*, *rovS,* and *stk1* genes showed no difference between the isolates, a variation in the *covR* gene was observed. In the HH/LC variant, the *covR* sequence contained a 3-bp deletion, which eliminates a valine that was encoded at position 31 (LH/HC = 20′-LELLHEGYDVVVETNGRE-37′ vs. HH/LC = 20′-LELLHEGYDVV_ETNGRE-36′). The published sequence of the serotype Ia genome strain A909 is identical to that of the LH/HC variant.
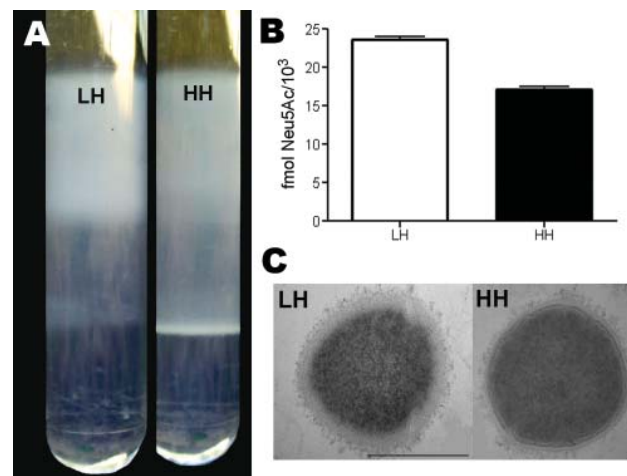

Figure 2. A) Buoyant density analysis of the low hemolytic (LH) and high hemolytic (HH) strains, exhibiting lower and higher buoyant density, respectively. B) Quantification of group B streptococci sialic acids expressed as fmol N-acetylneuraminic acid/1,000 CFUs of the LH and HH phenotypes. Error bars indicate SEM. C) Transmission electron microscopy of LH and HH phenotypic variants.

Sequence Variation in *covR* and Phenotypic Variation

To confirm that the *covR* 3-bp deletion contributed to the observed phenotypic changes in the HH/LC strain, we expressed the *covR/S* locus from the HH/LC variant on an expression plasmid in the LH/HC variant and GBS strain NEM316. Introduction of the mutated *covR/S* locus transferred the phenotypic appearance of the HH/LC variant to both the LH/HC and NEM316 strains, which resulted in increased hemolytic activity with increased pigmentation (Figure 3, panel A) and decreased reaction in the CAMP testing (Figure 3, panel B). In addition, encapsulation, measured by mean production of sialic acid, was reduced in the LH/HC expressing the HH *covR/S* locus by 29% compared with the parent LH/HC strain (from 24 to 17 fmol N-acetylneuraminic acid/10^3 CFU). To validate that the observed phenotypic changes were the result of changed transcriptional regulation, reverse transcription–PCR was performed on RNA isolated from the wild-type and transformant strains. The results demonstrated that introduction of the HH *covR/S* locus in either the LH/HC or NEM316 background reduced expression of the *cfb* gene (encoding CAMP factor) and increased expression of the *cyl* genes (Figure 3, panel C).

Bacterial Growth Rate

In culture media the HH/LC phenotypic variant grew markedly faster than the LH/HC variant (Figure 4, panel A) and even outgrew the LH/HC isolate when both phenotypes were cultured together (Figure 4, panel B). Both phenotypes showed a faster growth rate than the 2 control isolates (samples from vaginal colonization and neonatal sepsis patients), with division times of 35 and 45 minutes, respectively (data not shown).

Cytokine Induction

We further investigated the potential of the 2 variants to induce proinflammatory responses in human cells. Stimulation of PBMCs from different donors showed that both GBS variants induced IL-1β and TNF-α, but no overall difference was noted between the 2 isolates. Because β-h/c has previously been shown to be a potent inducer of IL-8 and β-h/c expression increases 4-fold in parallel with growth rate (*25*), we expected that a difference in IL-8 responses would be greatest when live HH/LC and LH/HC strains were used as stimuli. Indeed, live HH/LC bacteria induced significantly higher IL-8 levels than did the LH/HC isolate (p = 0.03) (Figure 5).

Murine Toxic Shock Model

Virulence of the 2 isolates was tested in a murine toxic shock model, by injecting each mouse intraperitoneally with $5–7 \times 10^7$ CFU. Although all mice rapidly became bacteremic after inoculation, the bacterial load in blood
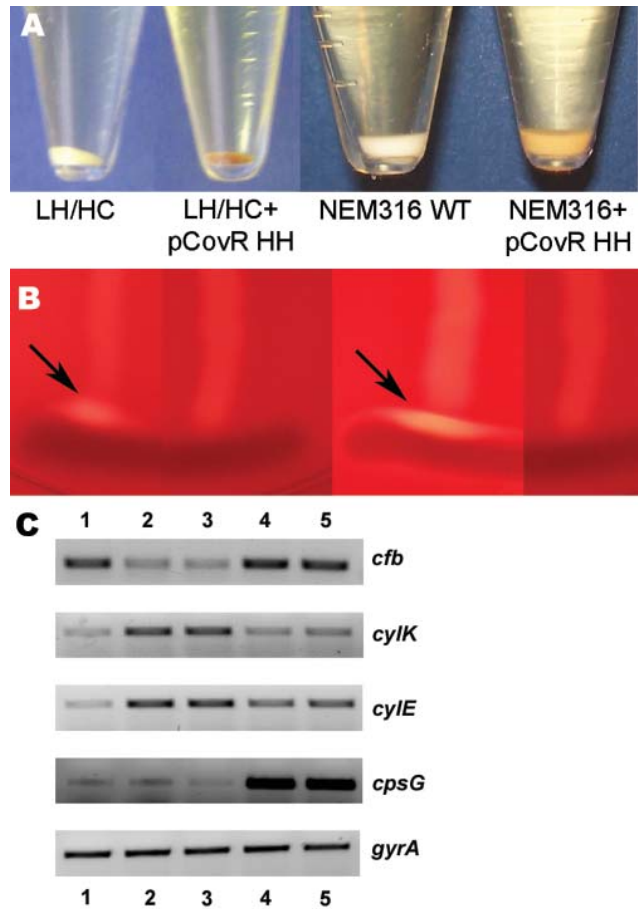

Figure 3. A) Difference in pigmentation of the low hemolytic (LH)/high encapsulation (HC) and NEM316 wild-type (WT) strains and their corresponding transformants expressing the *covR/S* locus of the high hemolytic (HH)/low encapsulation (LC) variant after overnight culture. B) CAMP testing with strains displayed according to panel A. The LH/HC variant and the NEM316 strain display a stronger reaction (arrows) than their corresponding transformants. C) Semiquantitative analysis of mRNA expression of CAMP factor (*cftb*), β-h/c (*cylK* and *cylE*) capsule (*cpsG*), and *gyrA* (housekeeping gene) using reverse transcription–PCR. Lane 1, LH/HC; lane 2, HH/LC; lane 3, LH/HC + pcovR HH; lane 4, NEM316 WT; lane 5, NEM316 + pcovR HH.

was significantly higher in mice infected with the LH/HC strain (p = 0.01) (Figure 6, panel A). Nevertheless, the HH/LC bacteria caused death significantly earlier (p = 0.0001) (Figure 6, panel B).

Resistance Toward Phagocytic Killing

The higher bacterial load of the LH/HC phenotype in the mice in comparison to the previously noted higher growth rate by the HH/LC phenotype in media (Figure 4) suggested an increased resistance toward host immune defense in this phenotype. Indeed, in human whole blood, the LH/HC variant exhibited a higher growth rate than
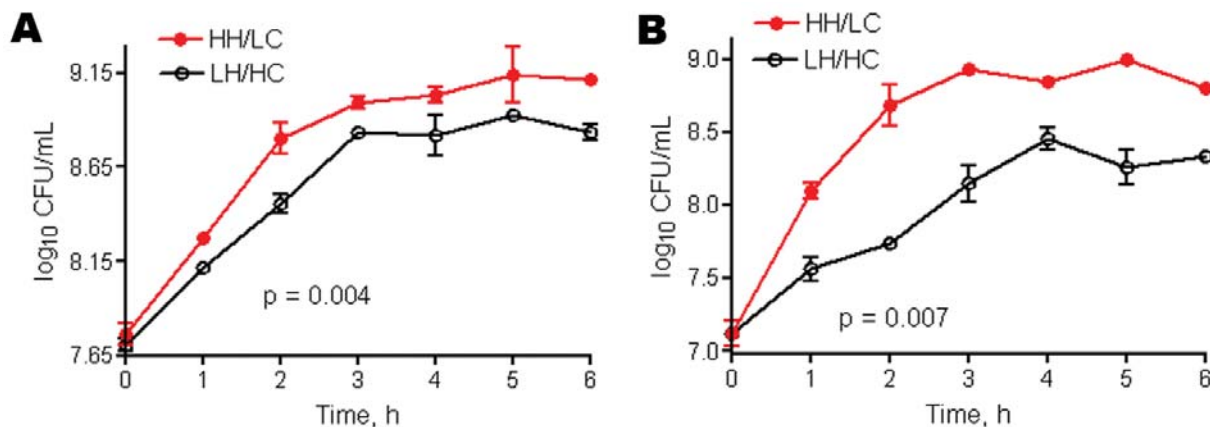
Figure 4. Growth curve of group B streptococcal variants, i.e., low hemolytic (LH)/high encapsulation (HC) and high hemolytic (HH)/low encapsulation (LC), in Todd-Hewitt broth plus 1.5% yeast cultured in a separate tube (A) or together in the same tube (B). Graph presented as mean + SD.

HH/LC (data not shown). We therefore further assessed these findings in several types of bactericidal assays. In a human whole-blood killing assay, the LH/HC phenotypic variant showed a higher survival index than the HH/LC variant at 2 different inocula (p = 0.03) (Figure 7, panels A and B). A similar survival advantage for the LH/HC variant against whole-blood killing was also observed in coculture assays in which both variants were used (data not shown). In an opsonophagocytic assay with purified neutrophils and complement, the LH/HC isolate again demonstrated a higher survival index than the HH/LC phenotype (p = 0.03) (Figure 7, panel C). The LH/HC strain also demonstrated greater intracellular survival within neutrophils compared with the HH/LC variant (p = 0.0012) (Figure 7, panel D).

We next sought to determine whether the enhanced resistance to bactericidal clearance would correspond to increased virulence in a low-dose infection model. Consistent with the high-dose sepsis model, recovery of bacteria from the bloodstream at 6 h postinfection was significantly higher in mice infected with LH/HC isolates than in those infected with the HH/LC phenotype (Figure 7, panel E). However, in contrast to high-dose challenge, in which death was accelerated in mice infected with the HH/LC variant, the increased resistance of the LH/HC against host phagocytic killing translated into sustained bacteremia and greater lethality (80% vs. 20%) in the lower dose infection model (Figure 7, panel F).

Discussion

The rate of invasive GBS in nonpregnant adults is increasing, and most cases are found in elderly persons and those with underlying diseases (26). This study is based on a rare case of toxic shock syndrome and necrotizing fasciitis in an immunocompetent man without apparent

risk factors. The GBS colonies obtained from the same tissue culture differed in phenotypic properties associated with 2 known GBS virulence factors, β-h/c cytotoxin and the exopolysaccharide capsule. A similar case was reported by Sigge et al. (27); they described a case of neonatal sepsis caused by GBS in which hemolytic and nonhemolytic colonies were displayed. Notably, only the hemolytic strain could be isolated from the maternal vaginal tract. The observation of GBS isolates of the same clonal origin, but with varying phenotypes, as described in our study and that of Sigge et al. (27) supports the concept of differential expression of certain virulence factors, either during the process of colonization or during infection of specific anatomic sites. These phenotypic changes may occur in response to selective pressures exerted by the host immune response, providing the pathogen a survival benefit. We therefore explored sequence differences in
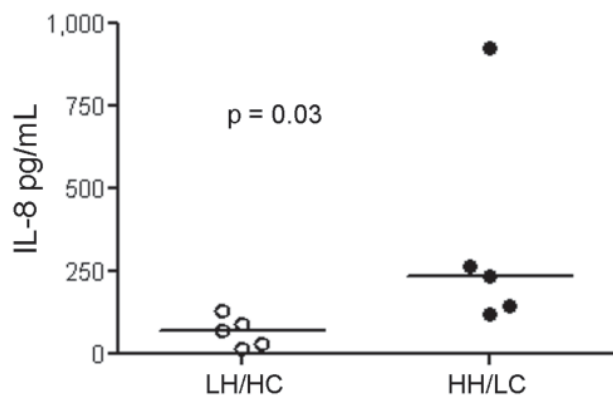

Figure 5. Interleukin (IL)– 8 induction in human peripheral blood mononuclear cells (PBMCs) (n = 5) using live bacteria. IL-8 concentration measured in cell culture supernatants of PBMCs were after exposure to live high hemolytic (HH)/low encapsulation (LC) and low hemolytic (LH)/high encapsulation (HC) bacteria. Horizontal lines indicate the median.
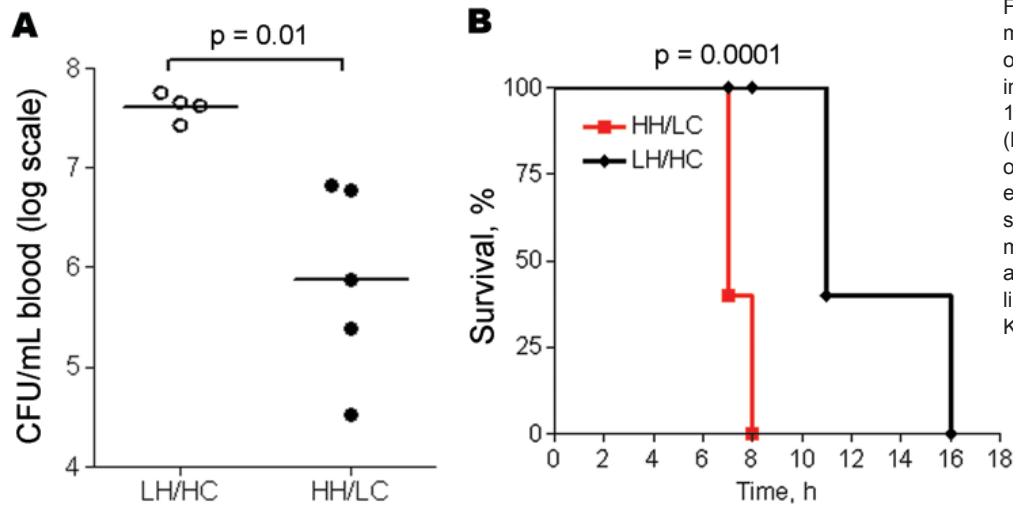
Figure 6. Murine toxic shock model (high inoculum). Groups of 5 mice were inoculated intraperitoneally with 5–7 × 10⁷ CFU of low hemolytic (LH)/high encapsulation (HC) or high hemolytic (HH)/low encapsulation (LC) group B streptococcal isolates per mouse. A) Level of bacteremia assessed after 6 h. Horizontal lines indicate the median. B) Kaplan-Meier survival plot.

potential genetic clusters of this phenotypic variation and also investigated the functional differences between the 2 phenotypes. We demonstrated that the 2 isolates have distinct phenotypic characteristics but are of the same clonal origin. Moreover, our findings indicate that these variations in phenotypic appearance are associated with significant differences in resistance to host phagocytic killing and in the clinical course of experimental infection. Finally, we provide indications that this phenotype switch may occur due to a mutation in an important regulatory gene (*covR*).

In our clinical pair of phenotypic variants, pigment production and hemolytic activity was reduced (but not eliminated) in 1 variant. However, no mutations in the *cyl* genes, the operon encoding genes required for β-h/c production, were identified. Sequence analysis of several regulatory genes showed that the HH/LC phenotype contained a 3-bp deletion in the *covR* gene. Previous studies have shown that complete deletion of the GBS *covR/S* may result in up-regulation of *cyl* genes involved in β-h/c expression (i.e., hemolytic activity), down-regulation of genes in the GBS *cps* operon for capsule expression, reduced survival in serum, and reduced virulence in animals (*28,29*). Additionally, Δ*covR/S* mutants show reduced CAMP activity, increased adherence to epithelial cells, and increased β-galactosidase activity (*28*); these changes illustrate the influence of *covR/S* on multiple genes involved in phenotypic, virulence, and biochemical properties of GBS.

To explore whether the 3-bp deletion in *covR* harbored by the HH/LC mutant may contribute to the observed phenotypic variation between the 2 clonal isolates, we expressed the *covR/S* locus from the HH/LC in the LH/HC variant, as well as the NEM316 GBS genome strain. Indeed, overexpression of the *covR/S* HH locus in these 2 genetic backgrounds resulted in a phenotype switch, i.e., in-

creased pigmentation associated with increased hemolytic activity, combined with decreased capsule production (as estimated by sialic acid quantitation) and CAMP reactivity. As demonstrated by reverse transcription–PCR, these observed phenotypic changes were paralleled by the expected changes in the mRNA transcripts for genes encoding β-h/c (*cylK, cylE*), CAMP factor (*cfb*), and *cps*G (an enzyme within the capsule biosynthetic operon).

Mutations in the related *covR/S* system of group A streptococci are induced under selective pressure of the innate immune system and contribute to the pathogenesis of invasive infection caused by strains of the M1T1 serotype, which is associated with necrotizing fasciitis and toxic shock syndrome (*30,31*). Future detailed genetic, transcriptional and mutational analysis of GBS invasive versus colonizing disease will be required to determine whether a similar paradigm exists in GBS.

Functional analyses of our clinical isolates showed that the HH/LC phenotype had a more rapid growth rate in culture media. Production of major GBS virulence factors (e.g., β-h/c, β-C protein) increases greatly with higher growth rate (*25,32*). In agreement with these results, the HH/LC phenotype induced a significantly higher release of the proinflammatory chemokine IL-8 than did the LH/HC phenotype. Considering the strong association between IL-8 serum levels and severity of sepsis (*33,34*), as well as the previously reported contribution of the β-h/c to the severe manifestations of septicemia in animal models (*13,35–37*), we compared the isolates in an in vivo sepsis model. Indeed, in a high-dose sepsis model in mice, the HH/LC phenotypic variant was associated with accelerated death, although blood CFU levels were lower than observed with the LH/HC variant. We hypothesize that the accelerated death of mice infected with HH/LC isolates is a result of an overwhelming inflammatory response. The higher CFU levels
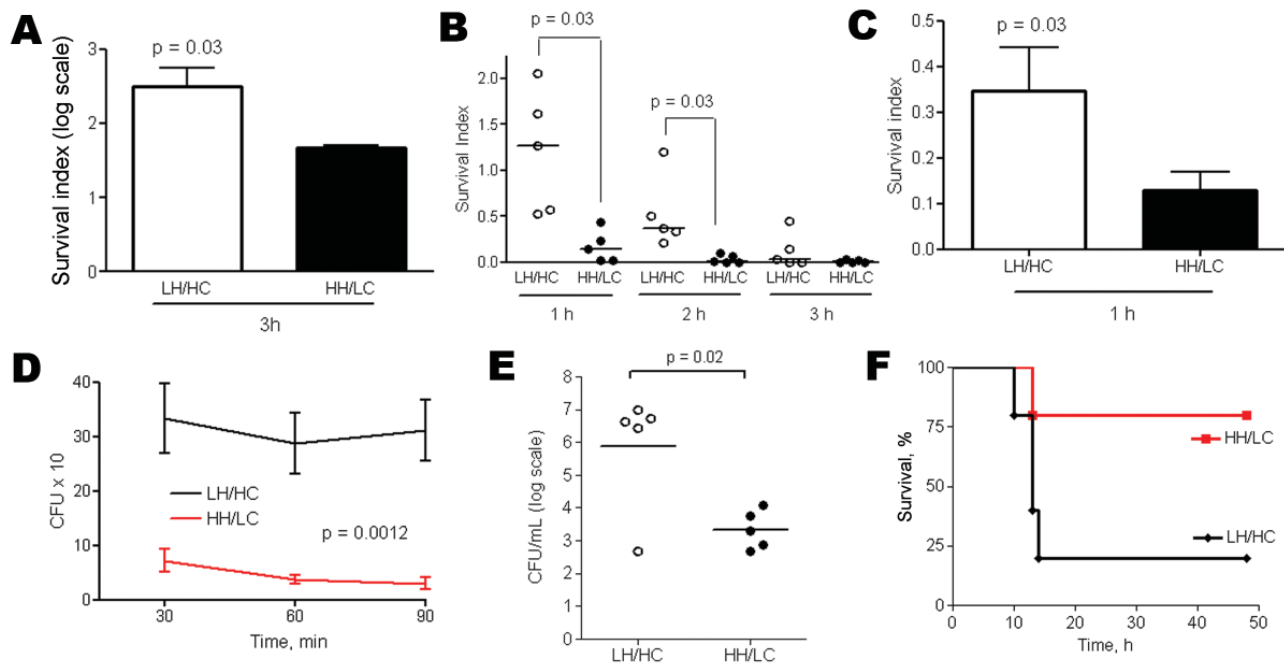
Figure 7. A) Human whole-blood killing assay after 3 h incubation, using 100 CFU bacteria in 100 mL phosphate-buffered saline (PBS) and 300 mL blood. Error bars indicate SEM. B) 100 CFU bacteria in 100 μL PBS and 1,000 μL blood. Survival index is calculated as follows: (CFU at the end of the assay)/(CFU at t = 0 h). Horizontal lines indicate the median. C) Opsonophagocytic killing assay after 1 h incubation, using a multiplicity of infection of »1:1 (CFU 10^6/μL: neutrophils 10^6/μL) and 10% volume serum. Error bars indicate SEM. D) Intracellular survival assays in neutrophils after 30, 60, and 90 min of extracellular antimicrobial drug exposure. Error bars indicate SEM. E) Murine model for invasive disease (low inoculum). Groups of 5 mice were inoculated intraperitoneally with 6–8 × 10^6 CFU of low hemolytic (LH)/ high encapsulation (HC) or high hemolytic (HH)/low encapsulation (LC) group B streptococcal isolates per mouse. Levels of bacteremia were assessed after 6 h. Horizontal lines indicate the median. F) Kaplan-Meier survival plot.

of the LH/HC variant, on the other hand, may be associated with their increased resistance to phagocytic clearance, as comprehensively investigated in 4 different experimental models, i.e., whole blood, neutrophils and complement, intracellular survival in neutrophils, and blood collection in the mouse model. Because complete elimination of β-h/c by targeted mutagenesis is known to diminish GBS resistance to phagocytic killing (16) and to reduce blood survival in a variety of animal models (10,12,14,16), the results imply a simultaneous up-regulation of a factor with a more critical role in phagocyte resistance in the LH variant. The hyperencapsulation in the LH variant is in line with this reasoning, because the capsule is known to impair opsonophagocytosis (4) and likely contributes to the enhanced resistance of the LH phenotype in vitro and in vivo.

In summary, our data show that phenotypic variants with markedly different expression of prominent virulence factors can arise in the course of invasive GBS infection in humans. These bacterial subpopulations may contribute to different aspects of disease pathogenesis. In the case reported here, we could hypothesize that the HH/LC variant exerts toxin-mediated direct tissue injury and proinflammatory effects and that the LH/HC variant displays enhanced resistance to phagocytic clearance by virtue of increased capsule. The evolution of genetic switch mechanisms by the pathogen may allow it to conserve biochemical resources and synthesize the highest levels of capsule only under in vivo conditions of phagocyte selective pressure. The disadvantages of constitutive high-level capsule expression may lie in the known inhibitory effects of capsule on epithelial cell adherence (38) and the reduced growth rate of the LH/HC variant as observed in our studies in optimal culture media. The potential for GBS phenotype variants should be a consideration in the diagnostic microbiology laboratory and in future analysis of GBS molecular pathogenesis.

Dr Sendi is an internal medicine and infectious disease physician at the Clinic for Infectious Diseases, University Hospital, Bern, Switzerland. His research interests focus on both pathogenesis and clinical features of invasive GBS diseases in nonpregnant adults.

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Face Mask Use and Control of Respiratory Virus Transmission in Households

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Many countries are stockpiling face masks for use as a nonpharmaceutical intervention to control virus transmission during an influenza pandemic. We conducted a prospective cluster-randomized trial comparing surgical masks, non–fit-tested P2 masks, and no masks in prevention of influenza-like illness (ILI) in households. Mask use adherence was self-reported. During the 2006 and 2007 winter seasons, 286 exposed adults from 143 households who had been exposed to a child with clinical respiratory illness were recruited. We found that adherence to mask use significantly reduced the risk for ILI-associated infection, but <50% of participants wore masks most of the time. We concluded that household use of face masks is associated with low adherence and is ineffective for controlling seasonal respiratory disease. However, during a severe pandemic when use of face masks might be greater, pandemic transmission in households could be reduced.

Highly pathogenic avian influenza virus A (H5N1) continues to spread globally, posing a serious human pandemic threat. In the event of an influenza pandemic or other emerging respiratory disease such as severe acute respira-

tory syndrome (SARS), it is likely that antiviral drugs and vaccines will be in short supply or that delivery could be delayed. Therefore, nonpharmaceutical interventions such as mask use, handwashing, and other hygiene measures or school closure might be effective early control strategies. In contrast to pharmaceutical interventions, little is known about the effectiveness of nonpharmaceutical interventions in the community. A recent analysis gives estimates of the effect of school closure (1), and several prospective, randomized controlled trials of handwashing have been published (2–11). However, clinical trial data on the ability of face masks to reduce respiratory virus transmission in the community are limited to 1 published prospective trial, which showed lack of efficacy (12). In addition, adverse effects of wearing masks (particularly respirators) may affect compliance and effectiveness (13–15). Despite the lack of quantitative evidence, many countries have included recommendations in their pandemic plans on the use of face masks (16–18). We present the results of a cluster-randomized household study of the effectiveness of using face masks to prevent or reduce transmission of influenza-like illness (ILI).

Methods

A prospective, cluster-randomized trial of mask use in households was conducted during the 2 winter seasons of 2006 and 2007 (August to the end of October 2006 and June to the end of October 2007) in Sydney, Australia. Enrollment in the study was restricted to households with ≥2 healthy adults ≥16 years of age; the adults had known exposure within the household to a child with fever and respiratory symptoms. Suitable households were identified at a pediatric health service comprising the emergency department of a pediatric hospital and a pediatric primary care practice in Sydney, New South Wales, Australia. The study protocol was approved by the local institutional review board.

Randomization and Intervention

Participating households were randomized to 1 of 3 arms by a secure computerized randomization process: 1) surgical masks (3M surgical mask, catalogue no. 1820; St. Paul, MN, USA) for 2 adults, to be worn at all times when in the same room as the index child, regardless of the distance from the child; 2) P2 masks (3M flat-fold P2 mask, catalogue no. 9320; Bracknell, Berkshire, UK), for 2 adults, to be worn at all times when in the same room as the index child, regardless of the distance from the child; and 3) a control group (no masks used). The P2 masks used have an almost identical specification as N95 masks used in the United States (19). According to New South Wales Health guidelines, pamphlets about infection control were provided to participants in all arms. Study participants and trial staff were not blinded, as it is not technically possible to blind the mask type to which participants were randomized. However, laboratory staff were blinded to the arm of randomization. Figure 1 shows the flow diagram for the trial as suggested by CONSORT guidelines (20).

Recruitment and Follow-up

Children 0–15 years of age seeking treatment at pediatric health services with fever (temperature >37.8°C) and either cough or sore throat were identified by an electronic triage system. Parents or primary caregivers were approached in the waiting room, and that household was invited to join the study if all of the following criteria were satisfied: 1) the household contained ≥2 adults ≥16 years of age and 1 child 0–15 years of age; 2) the index child had fever (temperature >37.8°C) and either a cough or sore throat; 3) the child was the first and only person to become ill in the family in the previous 2 weeks; 4) adult caregivers consented to participate in the study; and 5) the index child was not admitted to the hospital.

If eligibility criteria were satisfied, adults from the household were enrolled in the study. Enrolled adults and
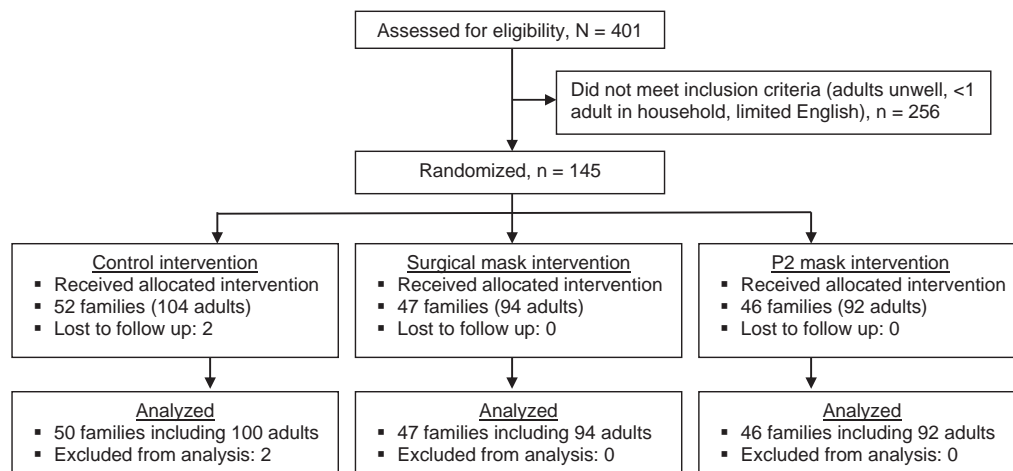

Figure 1. Flow diagram of recruitment for the prospective cluster–randomized trial, Sydney, New South Wales, Australia, 2006 and 2007 winter influenza seasons.

any siblings of the index child were then evaluated for respiratory symptoms and signs (fever, history of fever or feeling feverish in the past week, myalgia, arthralgia, sore throat, cough, sneezing, runny nose, nasal congestion, headache). If any of these symptoms were present, the family and household were excluded. Sociodemographic and medical information including influenza vaccination history (both the index child and participating adults) was obtained using a researcher-administered questionnaire. Medication use was also recorded. The index case-patient had combined nasal (each nostril) and throat swabs collected for multiplex reverse transcription–PCR (RT-PCR) testing. The household was randomized to 1 of the 3 arms, allocated the appropriate mask type, and educated about infection prevention. Formal fit testing of the P2 masks was not performed, but information pertaining to the correct method for fitting and disposing of the masks was provided. Over the next week, participants were contacted by telephone daily to determine if symptoms had developed and to record adherence to mask use throughout the day.

Each household was supplied with a thermometer to measure the temperature of symptomatic adult participants twice daily. If study staff determined that a participant had developed respiratory disease symptoms at follow-up, a home visit was conducted on the same day and the participant was swabbed and tested for respiratory viruses (see methods described below). Symptomatic participants were then followed up daily for 2 weeks.

Because all respiratory pathogens share similar transmission mechanisms—aerosol, droplet, and fomite spread (although the relative role of these factors may vary among different viruses and in different clinical situations)—we deliberately considered a broad definition of clinical cases consistent with a wide range of common respiratory viruses. Respiratory viruses detected in the study included influenza A and B, respiratory syncytial virus (RSV), adenovirus, parainfluenza viruses (PIV) types 1–3, coronaviruses 229E and OC43, human metapneumovirus (hMPV), enteroviruses, and rhinoviruses.

Adherence to face mask use was specifically monitored during each household follow-up. Measuring adherence and reasons for nonadherence is critical for evaluating the efficacy of mask use for reducing treatment and for providing practical advice on future use of face masks. Exit interviews with participants in the surgical mask and the P2 mask arms were conducted to gain further insights into adherence.

Sample Collection and Laboratory Testing

Rayon-tipped, plastic-shafted swabs were inserted separately into each participant's nostrils and pharynx, placed into viral transport media, and transported immediately to the laboratory or stored at 4°C if transport was delayed.

Nose and throat swabs of index children and adult participants with symptoms of respiratory illness were tested by using nucleic acid and a series of multiplex RT-PCR tests (21) to detect influenza A and B and RSV, PIV types 1–3, picornaviruses (enteroviruses or rhinoviruses), adenoviruses, coronaviruses 229E and OC43, and hMPV.

Case Definition

To include the broadest possible spectrum of clinical syndromes occurring among enrolled adults (22), during follow-up we defined ILI by the presence of fever (temperature >37.8°C), feeling feverish or a history of fever, ≥2 symptoms (sore throat, cough, sneezing, runny nose, nasal congestion, headache), or 1 of the symptoms listed plus laboratory confirmation of respiratory viral infection. The choice of a relatively broad clinical case definition was dictated by our interest in interrupting transmission of a broad range of respiratory viruses. Laboratory-confirmed cases during the follow-up were defined by the presence of ≥1 of the symptoms listed above plus laboratory detection of a respiratory virus.

Study Outcomes and Analysis

The primary study outcomes in enrolled adults were the presence of ILI or a laboratory diagnosis of respiratory virus infection within 1 week of enrollment. Given that we demonstrated some dual infections and that there may be a variable sensitivity of RT-PCR for different respiratory viruses, we included all incident infections in adults (by clinical case definition and laboratory testing) in the analysis. We also measured the time from recruitment to infection. Causal linking of the outcomes of ILI and adherence to use of face masks required consideration of the timing of both.

Analysis of primary outcomes was by intention to treat. We performed a multivariate Cox proportional-hazards survival analysis to study secondary outcomes and determine how time lag from recruitment to infection of a secondary case-patient was affected by explanatory covariates (23). Gaussian random effects were incorporated in the model to account for the natural clustering of persons in households (24). The day of infection was reconstructed from the day of symptom onset under the assumption that the incubation period was 1–2 days. To account for exposures that occurred before recruitment, the time when survival analysis started was defined as the maximum value between the day of recruitment minus the incubation period and the start of illness in the index case. (For example, assume a household recruited on day 0 and an incubation period of 2 days. If illness in the index case began on day –3, then the survival analysis began on day –2; if illness in the index case began on day –1, then the survival analysis began the same day.)

The following variables were included in the models: daily adherence to use of P2 or surgical masks,

number of adults in the household, number of siblings in the household, and index case ≤5 years of age. This analysis was performed using the survival package of the statistical software R (www.r-project.org). Comparisons among groups were made with the Fisher exact test for categorical variables. A 2-sided p value ≤0.05 was considered significant.

Power Analysis

Assuming a secondary attack rate in exposed adults of 20% and an intraclass correlation coefficient of 30%, we estimated that 94 adults would be needed in each arm of the study to show efficacy of ≥75% of P2 or surgical masks at 80% power and with a p value of 0.05. Our efficacy estimate was a conservative assumption based on observational data for the combined effects of all mask types during the SARS epidemic in Hong Kong (25).

Results

Study Population

We recruited 290 adults from 145 families; 47 households (94 enrolled adults and 180 children) were randomized to the surgical mask group, 46 (92 enrolled adults and 172 children) to the P2 mask group, and 52 (104 enrolled adults and 192 children) to the no-mask (control) group. Two families in the control group were lost to follow-up during the study. Characteristics of the families who participated are shown in Table 1, with no significant differences noted among the 3 arms.

Samples were collected from 141 children; respiratory viruses were detected in 90 (63.8%) children. In 79 (56.0%) of 141 cases, a single pathogen was detected: influenza A in 19/141 (13.5%); influenza B in 7/141 (4.9%); adenovi-

ruses in 7/141 (4.9%); RSV in 5/141 (3.5%); PIV in 8/141 (5.5%) (PIV-1 in 1/141 [0.70%]; PIV-2 in 2/141 [1.4%]; PIV-3 in 5/141 [3.5%]); hMPV in 8/141 (5.7%); and coronavirus OC43 in 3/141 (2.1%). Other viruses detected included picornaviruses in 22/141 (15.6%): rhinoviruses in 11/22 (50.0%); enteroviruses in 5/22 (22.7%) (enterovirus 68 in 1/5 [20.0%] and others in 4/5 [80.0%]); and uncharacterized nonsequenced picornaviruses in 6/22 (27.0%). An additional 11 children (7.8%) had dual or co-infection: 4 (2.8%) with adenovirus and rhinovirus, 2 (1.4%) with rhinovirus and coronavirus; and 1 each with influenza A and enterovirus, influenza A and PIV-2, influenza A and rhinovirus, RSV and enterovirus, and adenovirus and hMPV.

Adherence

Characteristics of the adherent versus nonadherent participants who were recruited are shown in Table 2; no significant differences were noted between the 2 groups except for the presence of ≥3 adults in the household. On day 1 of mask use, 36 (38%) of the 94 surgical mask users and 42 (46%) of the 92 P2 mask users stated that they were wearing the mask "most or all" of the time. Other participants were wearing face masks rarely or never. The difference between the groups was not significant (p = 0.37). Adherence dropped to 29/94 (31%) and 23/92 (25%), respectively, by day 5 of mask use (Figure 2).

Table 3 shows reported problems with mask use. There were no significant differences in difficulties with mask use between the P2 and surgical mask groups, but ≥50% reported concerns, the main one being that wearing a face mask was uncomfortable. Other concerns were that the child did not want the parent wearing a mask and the parent forgot to wear the mask. Additional comments made by some included that the mask did not fit well and that it was not

Table 1. Demographic characteristics of each household by arm of randomization in the study, Sydney, New South Wales, Australia, 2006 and 2007 winter influenza seasons.

Variable	Control group, no. (%), n = 50	Surgical mask group		P2 mask group	
		No. (%), n = 47	p value	No. (%), n = 46	p value
Living arrangement					
Reside in house	38 (76)	32 (68)	0.39	33 (72)	0.64
>4 persons in house	13 (26)	18 (38)	0.20	19 (41)	0.11
≥3 adults in house	8 (16)	11 (23)	0.36	12 (26)	0.23
Demographics					
Caucasian race*	28 (56)	20 (43)	0.18	17 (37)	0.06
Both adults work	28 (56)	25 (53)	0.78	27 (59)	0.79
Smoker in house	12 (24)	12 (26)	0.86	4 (9)	0.046
Index child fully immunized	45 (90)	45 (96)	0.28	39 (85)	0.44
Index child attends childcare	37 (74)	34 (72)	0.85	27 (59)	0.11
Influenza vaccination					
Index child	1 (2)	1 (2)	0.97	0	0.34
1 adult vaccinated	2 (4)	2 (4)	0.95	0	0.17
Duration of child sickness†	4	5		4	
Siblings reporting illness	3 (6)	1 (1)	0.34	0	0.09

*Information relates to the participating adult interviewed.
†Median no. days.

Table 2. Characteristics of adherent versus nonadherent mask wearers in the study, Sydney, New South Wales, Australia, 2006 and 2007 winter influenza seasons.*

Variable	Fully adherent mask users, no. (%), n = 30	Nonadherent mask users, no. (%), n = 156	p value
Living arrangement			
Reside in house	22 (73)	108 (69)	0.66
>4 persons in house	11 (37)	64 (41)	0.66
≥3 adults in house	3 (10)	43 (28)	0.04
Demographics			
Caucasian race†	10 (33)	29 (19)	0.07
Working adult	22 (73)	118 (76)	
Smoker in house			
Daily handwashing	14 (45)	54 (34)	0.21
Use of soap when handwashing	13 (43)	65 (42)	0.87
Index child fully immunized	15 (50)	69 (44)	0.56
Index child attends childcare	6 (20)	51 (33)	0.17
Influenza vaccination			
Index child	0	1 (0.5)	0.66
Adult 1	0	2 (1)	0.53
Adult 2	0	2 (1%)	0.53
Median days of child sickness	5	5	
Siblings reporting illness	0	1 (0.5)	0.66

*Adherence to mask use and handwashing measured by daily self-reports and exit interviews.
†Information relates to the participating adult interviewed.

practical to wear at meal time or while asleep. Some adults wore the mask during the day but not at night, even though the sick child was sleeping beside them in their bed.

Intention-to-Treat Analysis

ILI was reported in 21/94 (22.3%) in the surgical group, 14/92 (15.2%) in the P2 group, and 16/100 (16.0%) in the control group, respectively. Samples were collected from 43/51 (84%) sick adults, with respiratory viruses isolated in 17/43 (40%) sick adults. Viral pathogens were isolated from 6/94 (6.4%) in the surgical mask group, 8/92 (8.7%) in the P2 group, and 3/100 (3.0%) in the control group. In 10/17 laboratory-positive cases, the same respiratory virus was isolated in the adult and the child (surgical, 3/94; P2 group, 5/92; and control, 2/100). In 2 cases, the adult was the only person with a laboratory-confirmed virus (1 each from the P2 and surgical groups); in the remaining 5 adults, the virus detected in the child differed from that in the adult (surgical, 2; P2 group, 2; and control group, 1). No dual infections were detected in the adults. Intention-to-treat analysis by households and by participants showed no significant difference between the groups (Table 4).

Risk Factors for ILI

Under the assumption that the incubation period is equal to 1 day (the most probable value for the 2 most common viruses isolated, influenza [21] and rhinovirus [26]), adherent use of P2 or surgical masks significantly reduces the risk for ILI infection, with a hazard ratio equal to 0.26 (95% CI [confidence interval] 0.09–0.77; p = 0.015). No other covariate was significant. Under the less likely as-

sumption that the incubation period is equal to 2 days, the quantified effect of complying with P2 or surgical mask use remains strong, although borderline significant; hazard ratio was 0.32 (95% CI 0.11–0.98; p = 0.046). The study was underpowered to determine if there was a difference in efficacy between P2 and surgical masks (Table 5).

Discussion

We present the results of a prospective clinical trial of face mask use conducted in response to an urgent need to clarify the clinical benefit of using masks. The key find-
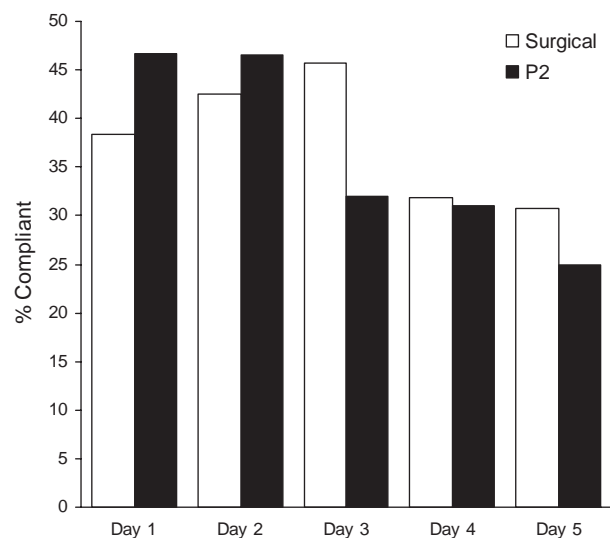

Figure 2. Compliance with face mask use by day over 5 consecutive days during the study, Sydney, New South Wales, Australia, 2006 and 2007 winter influenza seasons.

Table 3. Problems with face use reported by participants in the study, Sydney, New South Wales, Australia, 2006 and 2007 winter influenza seasons.

Reported problem	Surgical mask users, no. (%), n = 94	P2 mask users, no. (%), n = 92	p value
None	46 (49)	42 (46)	0.66
Uncomfortable	16 (17)	14 (15)	0.74
Forgot to wear it	8 (9)	8 (9)	0.96
Child did not like it	6 (6)	8 (9)	0.55
Other	18 (19)	20 (22)	0.66

ings are that ≤50% of participants were adherent with mask use and that the intention-to-treat analysis showed no difference between arms. Although our study suggests that community use of face masks is unlikely to be an effective control policy for seasonal respiratory diseases, adherent mask users had a significant reduction in the risk for clinical infection. Another recent study that examined the use of surgical masks and handwashing for the prevention of influenza transmission also found no significant difference between the intervention arms (*12*).

Our study found that only 21% of household contacts in the face mask arms reported wearing the mask often or always during the follow-up period. Adherence with treatments and preventive measures is well known to vary depending on perception of risk (*27*) and would be expected to increase during an influenza pandemic. During the height of the SARS epidemic of April and May 2003 in Hong Kong, adherence to infection control measures was high; 76% of the population wore a face mask, 65% washed their hands after relevant contact, and 78% covered their mouths when

sneezing or coughing (*28*). In addition, adherence may vary depending on cultural context; Asian cultures are more accepting of mask use (*29*). Therefore, although we found that distributing masks during seasonal winter influenza outbreaks is an ineffective control measure characterized by low adherence, results indicate the potential efficacy of masks in contexts where a larger adherence may be expected, such as during a severe influenza pandemic or other emerging infection.

We estimated that, irrespective of the assumed value for the incubation period (1 or 2 days), the relative reduction in the daily risk of acquiring a respiratory infection associated with adherent mask use (P2 or surgical) was in the range of 60%–80%. Those results are consistent with those of a simpler analysis in which persons were stratified according to adherence (online Technical Appendix, available from www.cdc.gov/EID/content/15/2/233-Techapp.pdf). We emphasize that this level of risk reduction is dependent on the context, namely, adults in the household caring for a sick child after exposure to a single index case.

Table 4. Intention-to-treat analysis used in the study*

	Control group, no. (%)	All masks			Surgical masks			P2 masks		
Data		No. (%)	RR (95% CI)†	p value†	No. (%)	RR (95% CI)†	p value†	No. (%)	RR (95% CI)†	p value†
By house	n = 50	n = 93			n = 47			n = 46		
ILI	12 (24)	25 (27)	1.12 (0.62–2.03)	0.84	15 (32)	1.33 (0.70–2.54)	0.50	10 (22)	0.91 (0.43–1.89)	0.81
By individual	n = 100	n = 186			n = 94			n = 92		
ILI	16 (16)	33 (18)	1.11 (0.64–1.91)	0.75	19 (20)	1.29 (0.69–2.31)	0.46	14 (15)	0.95 (0.49-1.84)	1
Laboratory confirmed infections										
Influenza A	0	3 (2)			1 (1)			2 (2)		
Influenza B	0	1 (0.5)			0			1 (1)		
RSV	1 (1)	1 (0.5)			0			0		
hMPV	0	0			0			0		
Adenoviruses	0	2 (1)			0			2 (2)		
PIV‡	1 (1)	1 (0.5)			1 (1)			0		
Coronaviruses§	1 (1)	0			0			0		
Rhinoviruses	0	5 (3)			3 (3)			2 (2)		
Enteroviruses	0	0			0			0		
Picornoviruses	0	1 (0.5)			0			1 (1)		
Total	3 (3)	14 (8)	2.51 (0.74–8.5)	0.19	6 (6)	2.13 (0.55–8.26)	0.32	8 (9)	2.90 (0.79–10.6)	0.12

*RR, relative risk; CI, confidence interval; ILI, influenza-like illness; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; PIV, parainfluenza virus.
†Reference group is the control group.
‡Types 1–3; 229E/OC43.
§Types 1–3.

Table 5. Estimates of hazard ratios for ILI in the study*

Variable	Global effect of mask use		Effect per mask type	
	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value
1-d incubation period				
Adherence to use of surgical or P2 mask†	0.26 (0.09–0.77)	0.015‡		
Adherence to use of surgical mask†			0.27 (0.06–1.24)	0.09
Adherence to use of P2 mask†			0.24 (0.05–1.08)	0.06
No. adults	1.07 (0.66–1.71)	0.80	1.06 (0.66–1.71)	0.80
No. siblings	0.86 (0.55–1.35)	0.52	0.86 (0.55–1.35)	0.52
Index patient <5 y of age	0.88 (0.41–1.89)	0.75	0.88 (0.41–1.89)	0.74
Frailty§		0.005‡		0.004‡
2-d incubation period				
Adherence to use of surgical or P2 mask†	0.32 (0.11–0.98)	0.046‡		
Adherence to use of surgical mask†			0.18 (0.02–1.38)	0.099
Adherence to use of P2 mask†			0.45 (0.12–1.62)	0.22
No. adults	1.13 (0.71–1.81)	0.60	1.14 (0.71–1.82)	0.59
No. siblings	0.80 (0.51–1.27)	0.34	0.80 (0.50–1.27)	0.34
Index patient <5 y of age	1.02 (0.46–2.24)	0.96	1.02 (0.47–2.25)	0.95
Frailty§		0.004‡		0.004‡

*ILI, influenza-like illness; CI, confidence interval.
†Time-dependent variable.
‡p<0.05 significant (indicates that the outcome for 1 person is correlated with the outcome of other persons in the household).
§This term measures if the clustering of subjects in households is relevant to quantify the risk of ILI infection.

We urge caution in extrapolating our results to school, workplace, or community contexts, or where multiple, repeated exposures may occur, such as in healthcare settings. The exact mechanism of potential clinical effectiveness of face mask use may be the prevention of inhalation of respiratory pathogens but may also be a reduction in hand-to-face contact. Our study could not determine the relative contributions of these mechanisms.

In our study, fit testing for P2 masks was not conducted because this is unlikely to be feasible in the general community during a pandemic. As such, we felt it was more appropriate to determine the efficacy of non–fit-tested masks. We found no difference in adherence between P2 and surgical masks, an important finding, as there is a common belief among healthcare workers that P2 masks are less comfortable. The size of the study did not permit conclusive comparison of the relative efficacy of P2 masks and surgical masks. Given the 5- to 10-fold cost difference between the 2 mask types, quantifying any difference in efficacy between surgical masks and particulate respirators remains a priority that needs to be addressed by a larger trial.

A possible limitation of the study is that some adults may have been incubating infection at the time of enrollment. However, this effect would have biased the results toward the null in the intention-to-treat analysis. The survival analysis explicitly accounted for the existence of a fixed incubation period and incubating infections at the time of enrollment. A potential alternative study design would be to enroll participants from asymptomatic households, do follow-up for development of infection, and then immediately intervene with masks. For such a design, given that only 15%–20% of closely exposed adults will develop illness after exposure to an ill child, thousands of households (rather than hundreds) would be required to afford the same study power. In addition, such a design would have been fraught with underascertainment of incident infections and delayed implementation of mask intervention. We believe ours is a more efficient design. A further limitation is that some parents may have acquired infection outside the home. We identified 5 child–parent pairs with discordant viral infections. The randomization process should have ensured that outside exposure was equally distributed between arms, and this effect would have biased the results toward the null.

In retrospect, relying on laboratory-confirmed cases as the primary outcome may have been unrealistic for a study of this size. ILI in enrolled adults was 17.1%, but laboratory confirmation was modest; the virus was identified in only 34.7% of adult ILI cases (the rate of laboratory diagnosis in children was high at 63.8%). However, even intention-to-treat analysis using ILI outcome shows no significant difference between the groups. We used self-reporting to determine adherence; previous research indicates that patient self-reporting is more reliable than judgments by doctors or nurses when compared against urine drug levels (30). In addition, the significant association between adherence and clinical protection provides internal validation of self-reporting as a measure.

An important aspect of this study is that we included respiratory viruses other than influenza. Although these viruses may differ in their relative dependence (accurate quantitation of this relativity is uncertain for the various viruses) on different transmission mechanisms (i.e., large

droplet, aerosol, or fomite), all are transmitted by the respiratory route. Therefore, face mask use should have some effect on virus transmission (e.g., interference with hand-nose contact), given that participants in all arms of the study received the same infection control advice. In addition, we argue that assessing multiple respiratory viruses allows our results to be generalized more broadly to other infections, including new respiratory viruses that may emerge in the future. Conversely, the low rate of confirmed influenza A or B infection (18.4%) in the study could mean that our findings are not directly applicable to a scenario in which influenza predominates. If influenza is more likely than the other viruses in our study to be transmitted by the respiratory route, the prevalence of mixed infections would tend to bias our results toward the null. However, it is possible that a pandemic strain may have different transmission characteristics than seasonal strains as demonstrated by attack rates in different age groups in pandemics compared with seasonal outbreaks and by the detection of influenza virus in different clinical samples in human influenza virus A (H5N1) cases.

Results of our study have global relevance to respiratory disease control planning, especially with regard to home care. During an influenza pandemic, supplies of antiviral drugs may be limited, and there will be unavoidable delays in the production of a matched pandemic vaccine (*31*). For new or emerging respiratory virus infections, no pharmaceutical interventions may be available. Even with seasonal influenza, widespread oseltamivir resistance in influenza virus A (H1N1) strains have recently been reported (*32*). Masks may therefore play an important role in reducing transmission.

Acknowledgments

Dr MacIntyre is head of the School of Public Health and Community Medicine at the University of New South Wales, Australia, and professor of Infectious Diseases Epidemiology. Her research interests include the detailed understanding of the transmission dynamics and prevention of infectious diseases, particularly respiratory pathogens such as influenza, tuberculosis, and other vaccine-preventable infections.

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Nontuberculous Mycobacteria, Zambia

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Clinical relevance of nontuberculous mycobacteria (NTM) isolated from 180 chronically ill patients and 385 healthy controls in Zambia was evaluated to examine the contribution of these isolates to tuberculosis (TB)–like disease. The proportion of NTM-positive sputum samples was significantly higher in the patient group than in controls; 11% and 6%, respectively (p<0.05). NTM-associated lung disease was diagnosed for 1 patient, and a probable diagnosis was made for 3 patients. NTM-positive patients and controls were more likely to report vomiting and diarrhea and were more frequently underweight than the NTM-negative patients and controls. Chest radiographs of NTM-positive patients showed deviations consistent with TB more frequently than those of controls. The most frequently isolated NTM was *Mycobacterium avium* complex. Multiple, not previously identified mycobacteria (55 of 171 NTM) were isolated from both groups. NTM probably play an important role in the etiology of TB-like diseases in Zambia.

Tuberculosis (TB) is a problem of enormous dimensions in Africa, and *Mycobacterium tuberculosis* is the most important causative agent. However, in industrialized countries, nontuberculous mycobacteria (NTM) also play a key role in etiology of TB-like syndromes. In Africa, the contribution of NTM to such disease has been examined on a small scale only (*1–6*).

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Zambia is a country with historically high prevalence rates of TB. Patients with acid-fast bacilli (AFB)–positive sputum, or those with chest radiographic findings suggestive of active TB, who do not respond to general antimicrobial drugs, are generally presumed to have pulmonary TB. In general, these patients are treated empirically for 6 months with a combination of drugs recommended by the World Health Organization. However, several TB-like syndromes in Africa could be caused by NTM. Thus, inconclusive diagnosis of pulmonary TB would lead to overdiagnosis of TB and in some cases, to inappropriate treatment for NTM infections.

When NTM are isolated from a usually sterile site (e.g., blood, bone marrow, lymph nodes, synovial fluid), diagnosis of true disease is generally straightforward. However, when NTM are isolated from nonsterile sources, such as sputum or bronchoalveolar lavage samples, the diagnosis is less definitive, especially when the colony numbers are low or NTM are isolated from only 1 cultured specimen. Therefore, it is a challenge to differentiate true NTM lung disease from contamination and colonization. Thus, finding AFB by microscopy of respiratory specimens or by culture may pose a diagnostic problem for the clinician. In 1997, the American Thoracic Society (ATS), published useful criteria for determining the clinical relevance of NTM isolates (*7*). In 2007, the ATS guidelines were revised to include more lenient diagnostic criteria (*8*). Clinical criteria include a symptomatic patient with pulmonary symptoms, nodular or cavitary opacities on chest radiographs, or a high-resolution computed tomography scan that shows multifocal bronchiectasis with multiple small nodules. In addition, the microbiologic criteria comprise positive culture results from ≥2 separate sputum samples, or positive culture results from ≥1 bronchial wash or lavage, or a lung biopsy specimen with mycobacterial histopathologic features

(granulomatous inflammation or AFB) and positive culture for NTM, or lung biopsy specimen showing mycobacterial histopathologic features and ≥1 sputum or bronchial washing culture positive for NTM.

In a pilot study performed in 3 hospitals in Zambia in 2001, high rates of NTM culture–positive sputum samples were obtained (P.C.A.M. Buijtels et al., unpub. data). Therefore, we studied the clinical relevance of and risk factors for isolation of NTM from HIV-positive and HIV-negative patients with chronic productive cough and from randomly selected community controls in Zambia.

Materials and Methods

The study was conducted in St. Francis Hospital in the district of Katete in Zambia from August 2002 through March 2003. Informed consent was obtained from all patients and controls before enrollment. The study was reviewed and approved by the research ethics committee of the University of Zambia, the Central Board of Health, and the Ministry of Health in Zambia.

The study population was composed of adults (≥15 years of age) with chronic (defined as ≥2 weeks) signs and symptoms and a productive cough who were admitted to the department of internal medicine at the hospital. Most (96%) of the included patients had respiratory tract symptoms. The other 4% of the patients had skin infections/abscesses or lymphadenopathy.

For each eligible patient who consented to participate in the study, 2 healthy community controls were recruited randomly from the neighboring community. These controls were not matched for age or other characteristics of the patients. Nested within this case–control study, the characteristics of NTM-positive and NTM-negative persons were analyzed separately.

At the time of enrollment, patients and controls were interviewed in their own language, and their medical records were reviewed by using a standard form. A detailed physical examination was conducted. Chest radiographs were evaluated in a blinded manner in the Netherlands without any additional clinical information. Radiographs were scored for mediastinal adenopathy, cavitation, pleural and pericardial fluid, miliary pathologic changes, alveolar infiltration, interstitial pathologic changes, other lung pathologic changes, or no pathologic changes. Results of the scoring system were chest radiographs with no pathologic changes, pathologic changes not suggestive of TB, and pathologic changes consistent with TB. Over 3 consecutive days, sputum was collected from patients with a productive cough. Controls were asked to gargle with normal saline if they could not produce sputum. The first 2 sputum samples or gargle specimens were cultured for mycobacteria, and a third sample was stored at –20°C until used.

Laboratory Methods

Sputum or gargle specimens were divided into 2 equal parts: half was decontaminated with N-acetyl-L-cysteine–NaOH and half was decontaminated by using 6% sulfuric acid to compare these decontamination procedures for culture of mycobacteria (9). Specimens were cultured in Mycobacteria Growth Indicator Tubes (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) according to the manufacturer's instructions and guidelines reported by Master (10). Mycobacterium isolates were identified by using the Accuprobe culture confirmation test for the M. tuberculosis complex (Accuprobe; bioMérieux, Marcy l'Etoile, France) or by 16S rRNA gene sequencing (11). Serologic testing for HIV was performed by using a qualitative immunoassay (Determine HIV-1/2; Abbott Laboratories, Abbott Park, IL, USA) and the Vidas HIV DUO assay (bioMérieux).

Data Analysis

Data were entered into SPSS version 6 software (SPSS Inc., Chicago, IL, USA) and analyzed by using STATA version 8.0 (StataCorp., College Station, TX, USA). Student t-tests were used to assess different means between groups; proportions were compared by using χ^2 tests. Univariate odds ratios with 95% confidence intervals were calculated to assess associations of potential risk factors for NTM positivity. A stepwise backward regression approach was used for multivariate analysis.

Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters. Underweight was defined as a BMI <18.

Clinical Diagnosis of NTM Lung Disease

NTM lung disease was diagnosed if patients had respiratory symptoms, abnormal chest radiographic results suggestive of Mycobacterium infection, and 2 positive sputum cultures with the same NTM. Patients or controls with positive NTM cultures who did not meet these criteria were considered colonized.

Results

From August 2002 through February 2003, 180 patients and 385 controls were enrolled in the study. Two sputum samples were cultured from 154 patients and 383 controls, and from 1 sample of the remaining participants. The median age of the patients and controls was 35 years (range 16–80 years) and 30 years (range 15–78 years), respectively. Female participants represented 55% (99/180) of the patient group and 69% (265/385) of the control group. A total of 128 (71%) of the patients were HIV positive, and 87 (23%) of the controls were HIV positive. Statistically significant differences in age, sex, and HIV status were observed between patients and controls.

Culture Results for Patients

Of 180 patients, 60 (33%) had only *M. tuberculosis* isolates in their sputum samples, 12 (7%) had *M. tuberculosis* and NTM, and 19 (11%) had only NTM (Table 1). Microscopic results of Ziehl-Neelsen–stained smears of sputum samples were positive in 46 (67%) of 69 patients with *M. tuberculosis* isolates and in 1 patient with an NTM-positive culture.

Of the 31 NTM-positive patients, 29 had 2 consecutive sputum samples subjected to culture. In 22 of the 29 patients, only 1 of 2 cultures was positive. Four NTM-positive patients had 2 positive NTM cultures; 1 of these patients had pulmonary disease. *M. intracellulare* was isolated twice from 2 of these 4 patients, *M. avium* was isolated from both samples from 1 patient, and NTM of 2 species and *M. tuberculosis* was isolated from 1 patient. One of 2 sputum cultures from 3 other NTM-positive patients contained mycobacteria that could not be identified. Two of these 3 patients had *M. intracellulare* in 1 sputum sample; *M. porcinum* was isolated from 1 patient.

Case Reports for Patients with Suspected NTM Disease

Characteristics of the 4 patients with NTM isolates in both sputum samples and of the 3 NTM-positive patients from whom 1 of the 2 sputum samples contained mycobacteria that could not be identified are shown in Table 2. For 3 patients (1, 2, and 4), NTM-associated disease was suspected because of the combination of symptoms, positive cultures, and pathologic changes consistent with TB seen on chest radiographs. However, only 1 patient (patient 4) fulfilled the ATS criteria for NTM lung disease. *M. intracellulare* was isolated from all 3 of these patients.

The first patient (patient 4) was a 32-year-old HIV-positive man who reported having had a productive cough with hemoptysis for 17 weeks. He also was vomiting and had diarrhea. His BMI was 15. He had been treated for TB. Results of a chest radiograph were consistent with TB and showed alveolar infiltration and interstitial pathologic changes. His condition did improve after treatment with antimicrobial drugs (chloramphenicol and tetracycline), and he was again given treatment for TB. Mycobacteria were cultured from 2 sputum samples and identified as *M. intracellulare*. The patient died 5 weeks later.

The second patient (patient 1), who had *M. intracellulare* pulmonary disease, was a 55-year-old HIV-positive man. He was admitted because of a productive cough with hemoptysis for 17 weeks. Diarrhea was also reported; his BMI was 17. He had been receiving treatment for TB for 4 months. Sputum obtained before treatment was AFB negative. Radiographic investigation of the chest showed cavities and alveolar consolidation. No improvement was seen after he was treated with antimicrobial drugs (chloramphenicol, amoxicillin, gentamicin, and metronidazole). Sputum was examined again and was smear positive for AFB. The first sputum culture showed *M. intracellulare*. In the second sputum culture, the isolated mycobacteria could not be identified because of logistic reasons. The patient died 3 weeks later.

The third patient (patient 2), who had *M. intracellulare* pulmonary disease, was a 45-year-old HIV-positive woman who had had respiratory signs and symptoms for >1 year. She was known to have asthma. Physical examination found enlarged submandibular, supraclavicular, and axillary lymph nodes. Her BMI was 20. Alveolar infiltration was seen on a chest radiograph. Treatment with chloramphenicol was started. Culture of the first sputum sample showed mycobacteria that could not be identified; the second sputum showed *M. intracellulare*. Three days after admission, the patient was taken home by her family and was lost to follow-up.

Table 1. Culture results for 180 hospitalized chronically ill patients and 385 controls, Zambia, August 2002–March 2003*		
Results	Patients	Controls
Culture exclusively *Mycobacterium tuberculosis*, no. (%)	60 (33)	2 (0.5)
Culture *M. tuberculosis* and NTM, no. (%)	12 (7)	1 (0.3)
Culture exclusively NTM,† no. (%)	19 (11)	61 (16)
Culture NTM,‡ no. (%)	31 (17)	62 (16)
2 sputum or gargle samples cultured	154	383
2 sputum or gargle samples cultured from NTM-positive person	29 of 31 NTM-positive patients	62 of 62 NTM-positive controls
1 NTM-positive culture in NTM-positive person with 2 samples cultured	22 of 29 NTM-positive patients	61 of 62 NTM-positive controls
1 NTM-positive cultures in NTM-positive person with 2 samples cultured	4 of 29 NTM-positive patients	1 of 62 NTM-positive controls
2 NTM-positive cultures in persons with 2 samples cultured§	4 of 154 patients with 2 samples cultured	1 of 383 controls with 2 samples cultured

*NTM, nontuberculous mycobacteria.
†Proportion of patients with exclusively NTM was comparable with controls (p = 0.2).
‡NTM isolated with or without *M. tuberculosis*.
§Significantly more patients than controls had 2 sputum or gargle cultures positive for NTM (p<0.05).

Table 2. Data for 4 patients with NTM in 2 consecutive sputum samples and 3 NTM-positive patients from whom 1 of 2 sputum samples contained mycobacteria that could not be identified, Zambia, August 2002–March 2003*

Patient no.	Isolate from first sputum sample	Isolate from second sputum sample	Ziehl-Neelsen staining	Sex	Age, y	HIV status	Temp, °C	Duration, wk	Chest radiograph result	Died	BMI
1†	*Mycobacterium intracellulare*	AFB not identified	1+	M	55	+	34.6	17	Suspected TB	Yes	17
2†	AFB not identified	*M. intracellulare*	–	F	45	+	36.1	487	Suspected TB	No	20
3	*M. intracellulare*	*M. intracellulare*	–	M	43	+	36.3	3	No pathologic changes	No	19
4†	*M. intracellulare*	*M. intracellulare*	–	M	32	+	36.8	17	Suspected TB	Yes	15
5	AFB not identified	*M. porcinum*	–	F	50	–	36.6	8	No suspected TB	No	21
6	*M. avium*	*M. avium*	–	M	33	+	37.4	17	No radiograph	No	16
7	*M. avium*	*M. peregrinum, M. tuberculosis*	–	F	24	+	35.0	13	No pathologic changes	Yes	NK

*NTM, nontuberculous mycobacteria; Temp, temperature at time of enrollment; Duration, duration of symptoms from time of coming to the hospital to enrollment in the study; BMI, body mass index; AFB, acid-fast bacilli; TB, tuberculosis; NK, not known. All patients had respiratory symptoms.
†Patients assumed to have pulmonary NTM disease.

Culture Results for Controls

M. tuberculosis was cultured from sputum or gargle specimens from 3 (0.8%) of the 385 controls; in 1 of these 3 controls, NTM and *M. tuberculosis* were isolated (Table 1). In 61 (16%) controls, only NTM were isolated; this number was comparable with the proportion of patients among whom only NTM were isolated (11%; p = 0.2). In 61 of 62 NTM-positive controls, only 1 sputum or gargle specimen was culture positive for NTM. In 1 control, who was HIV negative, 2 mycobacteria, *M. porcinum* and an unknown *Mycobacterium* sp., were isolated. No chest radiographs suggestive of TB were observed for any of the controls.

From 383 controls, 2 sputum or gargle samples were cultured for *Mycobacterium* spp. Significantly fewer controls (1/383) than patients (4/154) had 2 sputum or gargle cultures positive for NTM (p<0.05).

Mycobacterium spp. Isolated

To compare the influence of the decontamination method on the yield of mycobacteria, we divided sputum or gargle samples from all patients and controls into 2 equal parts before decontamination (*9*). A total of 635 sputum samples were cultured from 180 patients, and 1,532 sputum or gargle samples were cultured from 385 controls. The results of the cultures are shown in Table 3. The number of NTM (72) isolated from 635 sputum samples of patients was significantly higher than the number of NTM (99) isolated from 1,532 sputum or gargle samples from controls (11% and 6%, respectively, (p<0.001).

Mycobacteria were isolated from 273 (43%) of 635 sputum samples from patients; *M. tuberculosis* isolates were found in 201 (74%) of the 273 positive sputum specimens, and NTM were found in 72 (26%). The most frequently isolated NTM was *M. intracellulare*, which was found in 12 specimens.

Mycobacteria were isolated from 104 (6.8%) of 1,532 sputum or gargle samples cultured from the controls. *M. tuberculosis* was found in 5 (4.8%) of the 104 positive cultures, and NTM were found in 99 (95%). The predominant NTM isolated from the controls were *M. avium* (5 specimens), *M. goodii* (4 specimens), and *M. peregrinum* (4 specimens).

A total of 55 (32%) of 171 NTM isolated from patients and controls were not identified because their 16S rDNA sequences were absent in the BLAST (National Center for Biotechnology Information, Bethesda, MD, USA, www.ncbi.nlm.nih.gov) database. These 55 NTM species were closely related to various *Mycobacterium* spp. such as *M. intracellulare*, *M. malmoense*, *M. fortuitum*, *M. smegmatis*, and *M. terrae*.

Table 3. Results from cultures of sputum samples taken from hospitalized chronically ill patients and controls, Zambia, August 2002–March 2003

Result	Patients, no. (%)	Controls, no. (%)
Negative	362 (57)	1,428 (93)
Mycobacterium tuberculosis	201 (32)	5 (0.3)
M. avium complex	15 (2)	5 (0.3)
M. intracellulare	12 (2)	0
M. avium	3 (0.5)	5 (0.3)
M. gordonae	4 (0.6)	0
M. peregrinum	2 (0.3)	4 (0.3)
M. goodie	1 (0.2)	4 (0.3)
M. porcinum	1 (0.2)	3 (0.2)
M. lentiflavum	1 (0.2)	0
Unknown *Mycobacterium* spp.	13 (2)	42 (3)
Other *Mycobacterium* spp.*	3 (0.5)	7 (0.5)
Unidentified acid-fact bacilli	32 (5)	34 (2)
Total no. sputum samples	635	1,532

*Other *Mycobacterium* spp. in patients were *M. fortuitum*, *M. neoaurum*, and *M. simiae*. Other *Mycobacterium* spp. in controls were *M. fortuitum*, *M. asiaticum*, *M. aurum*, and *M. conspicuum*.

Comparison of Persons with and without NTM in Sputum or Gargle Samples

The 93 patients and controls with NTM-positive cultures had different clinical and radiographic features than the 472 patients and controls without NTM in sputum samples (Table 4). These persons were more likely to report vomiting and diarrhea, were more often underweight (BMI<18), more often had general malaise, and their chest radiographs more frequently showed changes consistent with TB, such as consolidation and interstitial changes. HIV status and presence of *M. tuberculosis* in the sputum culture did not differ between the NTM-positive and NTM-negative groups. There were no significant differences between these groups in terms of age (mean 36.7 years vs. 34.8 years; p = 0.2), sex, smoking habits, alcohol use, and previous treatment for TB (Table 5). The percentages of farmers and of persons in both groups who used unboiled milk were comparable. Moreover, NTM-positive persons used tap water more often than NTM-negative persons (p = 0.004). A subgroup analysis, restricted to patients with NTM and patients without NTM in sputum, yielded similar results (data not shown).

Independent risk factors for NTM culture–positive sputum were determined by using multivariate analysis. Two factors, underweight (BMI<18) and use of tap water, were independently associated with having an NTM-positive sputum culture (Table 6).

Discussion

The purposes of this study were to compare the prevalence of NTM in sputum between hospitalized chronically ill patients and community controls and to determine the clinical importance of isolation of NTM. The proportions of patients and controls with positive sputum or gargle cultures for NTM were comparable (11% and 15%, respectively). However, the proportion of NTM-positive sputum samples was higher for patients than for controls (11% and 6%, respectively). This finding suggests that persistent NTM are associated with chronic illness in these patients. It is not known whether culture results were influenced by the method of obtaining specimens. A gargle specimen contains flora of the oropharyngeal mucosa, whereas a sputum sample contains flora of the lower airways. In the patient group, more persons were capable of producing sputum, which may have influenced the yield of positive NTM-positive cultures.

NTM lung disease was definitively diagnosed for 1 patient and probable diagnosis was made for 3 patients (nos. 1, 2, and 4; Table 2). *M. intracellulare* was isolated from the sputum samples of these HIV-positive patients. These patients had respiratory symptoms, and chest radiographs showed pathologic changes compatible with TB. Unfortunately, in 2 of these patients, 1 of 2 sputum samples with mycobacteria could not be identified because of contamination and reculture problems.

The combination of symptoms, positive cultures, and pathologic changes seen on chest radiographs are characteristics of NTM infection and suggestive of NTM pulmonary disease. However, the ATS criteria valid at the time of the study were not completely fulfilled because only 2 sputum samples were cultured for mycobacteria on consecutive days, instead of the 3 samples recommended. Furthermore, 2 of these patients suspected of having NTM lung disease had been treated for TB. Because these sputum samples were not tested with molecular amplification techniques for multidrug-resistant *M. tuberculosis*, the possibility that they had multidrug-resistant TB could not be excluded (*12,13*). Conversely, performance of these nucleic acid amplification tests is generally good for clinical respiratory specimens that are AFB smear positive but less so for specimens that contain fewer organisms or are AFB negative. Moreover, because sputum specimens were not cultured on solid medium, it was not possible to count the number of colony-forming units to distinguish colonization and infection from disease.

Many risk factors for NTM have been identified (*14–16*). In this study in a setting in Africa, HIV, sex, and age were not risk factors for NTM. The 2 risk factors for a positive NTM culture were being underweight and having consumed tap water. NTM are natural inhabitants of

Table 4. Clinical data for samples obtained from NTM-positive and NTM-negative persons, Zambia, August 2002–March 2003*				
Characteristic	NTM-positive samples	NTM-negative samples	p value	All samples
Persons, no. (%)	93 (16.5)	472 (83.5)	–	565
HIV positive, no. (%)	41 (45.6)	174 (38.5)	0.2	215 (39.7)
Mycobacterium tuberculosis, no. (%)	13 (14.0)	62 (13.1)	0.8	75 (13.3)
BMI, mean (SD)	20.2 (4.2)	20.8 (3.7)	0.2	20.7 (3.8)
Underweight, no. (%)	26 (29.6)	92 (20.6)	0.06	118 (22.1)
Vomited, no. (%)	8 (8.6)	18 (3.8)	0.04	26 (4.6)
Diarrhea, no. (%)	12 (12.9)	11 (2.3)	<0.001	23 (4.7)
Lymph nodes analyzed, no. (%)	21 (22.8)	146 (30.9)	0.1	167 (29.6)
Chest radiograph compatible with TB but culture negative for *M. tuberculosis*, no. (%)	5 (26.3)	28 (7.1)	0.003	33 (8.0)
Died, no. (%)	9 (9.7)	26 (5.5)	0.1	35 (6.2)

*NTM, nontuberculous mycobacteria; BMI, body mass index; TB, tuberculosis.

Table 5. Background characteristics of NTM-positive and NTM-negative persons, Zambia, August 2002–March 2003*

Characteristic	NTM positive, no. (%)	NTM negative, no. (%)	p value	All persons, no. (%)
Persons	93 (16.5)	472 (83.7)	–	565
Female	61 (65.6)	303 (64.2)	0.8	364 (64.4)
Age, y, mean (SD)	36.7 (13.1)	34.8 (14.6)	0.2	35.1 (14.4)
Farmer	48 (51.6)	245 (51.9)	1.0	293 (51.9)
Used tap water	23 (25.0)	62 (13.2)	0.004	85 (15.1)
Used unboiled milk	15 (16.1)	62 (13.1)	0.4	77 (13.6)
Smoker	5 (5.7)	41 (8.9)	0.3	46 (8.4)
Used alcohol	9 (10.3)	51 (11.2)	0.8	60 (11.0)
Hospitalized	31 (33.3)	149 (31.6)	0.7	180 (31.9)
Previously treated for TB	9 (9.7)	28 (5.9)	0.2	37 (6.6)

*NTM, nontuberculous mycobacteria; TB, tuberculosis.

municipal water systems and soil. A biofilm may form in the water distribution system and be a source of replicating NTM (*17*). Consequently, availability of clean tap water may introduce a serious danger, in particular, to immunosuppressed human populations. Therefore, tap water in Zambia should be tested for NTM.

In this study, patients and controls with NTM in sputum or gargle samples more often had symptoms and signs of general malaise, including diarrhea, vomiting, and being underweight, and chest radiographs for these NTM-positive persons more often showed pathologic changes than did those for NTM culture-negative persons. These symptoms and signs may not be specific for NTM infection, but they may reflect the patients' poor health in general.

Differences in geographic distribution of NTM species have been reported (*16,18,19*). The most commonly encountered NTM from clinical specimens in industrialized countries are *M. avium* complex (MAC) and *M. kansasii* (*20–24*). Despite limited studies conducted in Africa, the distribution of NTM is not known. In our study, the most commonly isolated NTM in patients and controls was *M. avium* complex. However, 32% of NTM found in both groups in Zambia have not been identified on a species level. This study indicates that the distribution of NTM in Africa may differ from that in Europe and the United

States. NTM in Africa may have diverged from NTM in industrialized countries. This hypothesis could be tested by extensive DNA sequencing of semiconserved genes such as those for RNA polymerase B and 65-kD heat-shock protein. Unidentified NTM colonize persons in Africa and can cause disease in some instances. The magnitude of this problem, in addition to the problem of TB, is unknown but deserves more attention.

Rates of NTM colonization and disease that have been reported vary in different areas. In North America and Europe, rates of colonization and disease in the general population range from ≈1–15/100,000 persons to 0.1–2 /100,000 persons, respectively (*20–23,25–28*). These rates are largely unknown for most countries in Africa. In South Africa, prevalence rates of NTM colonization of 1,400–6,700/100,000 persons have been reported (*29,30*). In gold miners in South Africa, rates of infection were 101/100,000 persons for NTM, 66/100,000 persons for *M. kansasii*, and 12/100,000 persons for *M. scrofulaceum* (*31,32*). Although numbers of cases were small, the estimated rate of colonization in our study in the patient population was 9% (14/154) and the rate of disease was ≈2% (3/154). Two sputum or gargle specimens were collected and cultured from 383 controls in our study. NTM were isolated from both specimens for 1 of 61 controls with ≥1 sample being

Table 6. Crude and adjusted risk factors for isolation of NTM from sputum samples, Zambia, August 2002–March 2003*

Factor	Univariate analysis, OR (95% CI)	Multivariate analysis, OR (95% CI)†
Hospitalized	1.1 (0.7–1.7)	NS
Age >25 y	1.3 (0.8–2.5)	NS
Sex (female)	1.1 (0.7–1.7)	NS
Underweight (BMI <18)	1.6 (1.0–2.7)	1.7 (1.0–2.9)
Mycobacterium tuberculosis infection	1.1 (0.6–2.0)	NS
Previous treatment for TB	1.7 (0.8–3.7)	NS
HIV positive	1.3 (0.8–2.1)	NS
Used tap water	2.2 (1.3–3.8)	2.0 (1.1–3.5)
Used alcohol	0.9 (0.4–1.9)	NS
Smoker	0.6 (0.2–1.6)	NS
Used unboiled milk	1.3 (0.7–2.3)	NS
Farmer	1.0 (0.6–1.5)	NS
Chest radiograph compatible with TB but culture negative for *M. tuberculosis*	4.7 (1.6–13.9)	NS

*NTM, nontuberculous mycobacteria; OR, odds ratio; CI, confidence interval; NS, not significant; BMI, body mass index; TB, tuberculosis.
†Stepwise backward elimination.

culture positive for NTM. This control was not suspected of having NTM pulmonary disease. The estimated rate of colonization in the general population on the basis of this result is 16% (61/383).

NTM probably play a role in the etiology of TB-like disease in Zambia. More extended studies, in terms of duration and size, will be needed to determine the true prevalence of NTM infection in Africa.

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Methicillin-Susceptible *Staphylococcus aureus* in Skin and Soft Tissue Infections, Northern Italy

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During February 2004–September 2006, familial clusters and sporadic cases of *Staphylococcus aureus* skin and soft tissue infections were observed in a suburban area near Milan in northern Italy. Molecular typing of the isolates showed an epidemic methicillin-susceptible *S. aureus* (MSSA) strain, *spa* type 005 and sequence type 22 that harbored Panton-Valentine leukocidin (PVL) genes. The first case-patients were neonates or mothers who had recently delivered in the local hospital. Examination of the medical records showed a cluster of postpartum mastitis and neonatal skin infections antedating the emergence of infections in the community. Nasal swabs of neonates, mothers, and hospital staff were positive for the epidemic MSSA. Hospital circulation of the strain was interrupted by implementation of infection control measures, although infections continued to occur in the community. The PVL-positive MSSA strain resembles typical community-acquired methicillin-resistant *S. aureus* in its ability to cause prolonged community and hospital outbreaks of skin infections.

Long established as a hospital pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA) is now present in the community as a major cause of skin and soft tissue infections (*1*). In the United States the community-acquired (CA)–MRSA clone designated USA300 has been identified in almost 50% of community-onset skin infections (*2*). In Europe, CA-MRSA infections appear to be less common than in the United States, although incidence is increasing (*3,4*) and CA-MRSA strains are more genetically diverse (*5*).

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Characteristically, most CA-MRSA strains contain Panton-Valentine leukocidin (PVL) genes, a bicomponent pore-forming toxin with the ability to lyse leukocytes (*6*), primarily associated with skin infections such as furunculosis and skin abscesses (*7*). Although clinicians are currently concerned primarily with CA-MRSA infections, methicillin-susceptible *S. aureus* (MSSA) infections can present with similar epidemiologic and clinical characteristics (*8,9*). In addition, the presence of PVL genes is not limited to MRSA nor is their presence a recent occurrence. Historical MSSA isolates, such as the "Oxford Staphylococcus" and phage type 80/81 strains that were pandemic in the 1950s and 1960s harbor PVL genes (*10,11*). Recently, PVL-positive MSSA strains have been associated with outbreaks of skin infections in Swiss schoolchildren (*12*), in a village in Germany (*13*), and in French soldiers operating in Côte d'Ivoire (*14*). According to a large multinational clinical trial, conducted outside the United States, PVL-positive *S. aureus* isolates are more likely to be MSSA than MRSA (*15*). The epidemiology of PVL-positive MSSA is not well known and the pathogenic potential is probably underestimated.

This report describes a large and prolonged community and hospital outbreak of skin and soft tissue infections caused by a PVL-positive MSSA strain. In a number of characteristics, the outbreak closely resembles outbreaks associated with CA-MRSA.

Methods

Setting of the Community Outbreak

During February 2004–September 2006, several family clusters of skin and soft tissue infections were observed in a suburban area south of Milan in northern Italy. The

patients lived in or near the town of Codogno and sought treatment at the outpatient clinic of the local hospital. In each of the family clusters, the first identified case was a newborn child or a mother who had recently delivered in the Codogno Hospital. In 2006, another cluster of skin and soft tissue infections was observed in young patients who lived in different households in the same suburban area.

Setting of the Hospital Outbreak

Medical records and microbiologic data regarding skin and soft tissue infections occurring in neonates or mothers examined in the Codogno Hospital from 2003 through 2005 were reviewed. At the time of the review, the hospital was a 220-bed facility, serving a community of ≈15,000 inhabitants. The maternity ward comprised 7 rooms with a total of 13 beds and was part of the Department of Obstetrics and Gynecology. The department had 45 staff members that included obstetricians, midwives, nurses, and support staff. Annually, ≈600 deliveries were performed. The newborn nursery was under the direction of the Department of Pediatrics and included 2 rooms, 1 room for changing and feeding the babies and the other room with 14 cribs for the neonates. The nursery staff included 9 neonatal nurses and 1 general nurse, but 16 additional staff members were shared across the Department of Pediatrics. The median length of stay of neonates in the nursery was 4 days. A follow-up visit was performed at a dedicated hospital clinic 10–15 days after discharge.

Microbiologic and Molecular Typing Methods

Bacteriologic specimens were obtained for culture from the largest infected body area with a sterile swab or needle and were processed in the microbiology laboratory of the Codogno Hospital according to standard methods. Swabs obtained from anterior nares were plated directly onto salt mannitol agar plates and blood agar plates without a preenrichment step. Plates were incubated for 24 h in ambient air and 5% CO_2, respectively. Identification of isolates and antimicrobial susceptibility tests were performed with an automatic system (Vitek 2; bioMérieux, Marcy l'Etoile, France). The susceptibility pattern was confirmed by the disk-diffusion method following Clinical and Laboratory Standards Institute guidelines (16).

Further molecular tests and genotyping were performed on all *S. aureus* isolates that had been stored. Bacterial DNA was prepared with a commercial kit (QIAamp DNA Mini Kit; QIAGEN GmbH, Hilden, Germany). Species identification and methicillin susceptibility were confirmed by a duplex PCR assay with primers targeting *nuc* and *mecA* genes, respectively. Detection of the presence of the genes *lukS-PV* and *lukF-PV* coding for the 2 subunits of the PVL toxin was obtained by PCR (17).

To analyze clonal relatedness of the strains, all available isolates were submitted to pulsed-field gel electrophoresis (PFGE) and to a sequence-based method that detects variations in the short sequence repeat (SSR) region of the protein A gene (*spa* typing) (18). For PFGE, total genomic DNA embedded in agarose plugs was digested with *Sma*I and separated by using a previously described method (19). Profiles of strains that differed by fewer than 3 bands were considered to belong to the same PFGE type (20). For *spa* typing, PCR amplification of the SSR region was performed according to the protocol of Shopsin et al. (18). Sequences were obtained and analyzed by using an Internet-based software Ridom Staph Type (www.ridom.de/spaserver). Selected isolates underwent multilocus sequence typing (MLST) according to the recommended method (21). The allelic profiles obtained were compared with those deposited in the MLST database (http://saureus.mlst.net).

Screening for *S. aureus* Carriage

To ascertain the spread of *S. aureus* in the maternity ward and nursery of the hospital, we performed screening for *S. aureus* nasal colonization on neonates, mothers, and medical and nonmedical staff who worked in the Departments of Pediatrics and Obstetrics and Gynecology. Two separate surveys were performed, the first in July 2005 and the second in December 2005, after implementation of enhanced infection control measures.

Results

Skin and Soft Tissue Infections in the Community

Five familial clusters of skin and soft tissue infections, involving 2–5 family members, were observed in the Codogno area. The first case in each cluster occurred in 2004 or early 2005, but infections in other household members or recurrent infections continued to be observed until 2006. In all families, the onset of infection was associated with a neonate born in the Codogno Hospital or a mother who had recently delivered in the same hospital.

Furunculosis and abscesses were the most common clinical features and relapses were common (Table 1). Furunculosis of the prepuce developed in a neonate in family 1 four days after birth in the Codogno Hospital in February 2004. His father sought treatment for recurrent subcutaneous axillary abscesses later in 2004 and in 2005, and a leg abscess developed in his mother in September 2006.

The mother in family 2 sought treatment for an infection in the vulva, groin, and inner thighs in August 2004, ten days after delivering at the Codogno Hospital. Several recurrences of abscesses in the same areas occurred as well as in this patient's left buttock and leg until 2006. In 2005, furunculosis developed in the father on his nose and scalp,

and recurring pustules developed on the inner thighs of 2 siblings over 2 months.

In family 3, a subcutaneous facial abscess developed in the mother in January 2005, two months after she delivered at the Codogno Hospital; subsequently, a vulvar abscess and recurrent abscesses of the right leg continued to develop in this patient until 2006. The child had a leg abscess in June 2006.

Family cluster 4 involved 5 persons: a neonate, a sibling, both parents, and the maternal grandmother. Pustules developed on the neck and groin of the neonate 4 days after birth at the Codogno Hospital in January 2005 and subsequently, subcutaneous abscesses developed in the axilla and forearm. Over the same time multiple subcutaneous abscesses developed in the axillae and on the forearms and legs of both parents and the elder sibling. The grandmother had a facial abscess in July 2005, and the father contracted furunculosis of the left forearm in September 2006.

Family cluster 5 involved both parents of a child who was born in the Codogno Hospital in December 2004 but

Table 1. Characteristics of patients with community-acquired MSSA skin and soft tissue infections, their treatment, and molecular typing of the isolates, northern Italy, 2004–2006*

Patient	Age, sex	Site of infection†	Type of infection†	Antimicrobial drug treatment†	Drainage	Presence of PVL genes	PFGE type	*spa* type	ST
Family clusters									
Cluster 1									
P 1	32 y, F	Leg	Abscess	None	Spontaneous	NA			
P 2	33 y, M	Axilla	Abscesses	AMC, CIP	None	NA			
P 3	4 d, M	Prepuce	Pustules	GEN	None	NA			
Cluster 2									
P 4	30 y, F	Vulva, thighs	Pustules, abscesses	AMC, LFX, TEC	None	+	A	t005	22
P 5	33 y, M	Nose, scalp	Pustules	AMC	None	NA			
P 6	14 mo, F	Thigh	Pustules	CLI	None	NA			
P 7	14 d, F	Thigh	Pustules	CLI	None	+	A	t005	ND
Cluster 3									
P 8	32 y, F	Face, vulva, leg	Abscesses	AMC, CIP, LFX	None	+	A	t005	22
P 9	25 mo, F	Leg	Abscess	None	Spontaneous	NA			
Cluster 4									
P 10	34 y, F	Axilla, forearm, leg	Abscesses	AMC	None	NA			
P 11	35 y, M	Axilla, forearm, leg	Abscess, furuncles	AMC, LFX	Surgical	+	A	t005	ND
P 12	4 d, M	Neck, groin, axilla,	Pustules, abscesses	AMC, AMC	Spontaneous	+	A	t005	22
P 13	3 y, M	Forearm	Abscesses	AMC	None	NA			
P 14	65 y, F	Axilla, forearm, leg, face	Abscess	None	None	NA			
Cluster 5									
P 15	33 y, F	Face, leg, axilla	Pustules, abscess	AMC	Surgical	NA			
P 16	36 y, M	Thigh	Furuncles, abscess	AMC	None	NA			
Sporadic cases									
P 17	64 y, F	Axilla	Abscess	CIP	None	+	A	t005	22
P 18	7 mo, F	Arm	Pustules	AMC	None	–	F	t159	ND
P 19	9 y, F	Axilla	Furuncles	AMC	None	–	F	t159	ND
P 20	12 mo, M	Groin	Abscess	AMC	None	+	A	t005	ND
P 21	8 y, F	Leg	Abscess	AMC	None	–	G	t445	ND
P 22	18 mo, M	Forearm	Furuncles	AMC	None	+	A	t005	ND
P 23	12 mo, F	Buttock	Abscess	AMC	Spontaneous	+	A	t005	ND
P 24	20 mo, F	Thigh	Abscess	AMC	None	+	A	t005	22
P 25	8 y, M	Arm, chest	Abscesses	AMC	Surgical	+	A	t005	ND
P 26	11 y, M	Face, eye	Abscess, conjunctivitis	AMC	None	+	A	t005	22

*MSSA, methicillin-susceptible *Staphylococcus aureus*; PVL, Panton-Valentine leukocidin; PFGE, pulsed-field gel electrophoresis; ST, sequence type; NA, isolate not available; AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin; GEN, gentamicin (topical); LFX, levofloxacin; TEC, teicoplanin; CLI, clindamycin (topical); ND, not determined.
†When >1 site or type of infection or antibimicrobial agents are indicated, they refer to different infection episodes.

who did not experience skin infections. Facial pustules developed in the mother in February 2005, two months postpartum. Subsequently, she was treated for a leg abscess and axillary furunculosis. In March 2006, a subcutaneous abscess developed in the thigh of her husband in March 2006.

All cases with a bacteriologic diagnosis were caused by MSSA that showed a distinct susceptibility pattern: resistant to penicillin and gentamicin and susceptible to oxacillin, erythromycin, tetracycline, rifampicin, and ciprofloxacin. Nasal swabs were performed in 7 patients from the clusters and were positive for MSSA in 6.

During March–June 2006, eleven additional cases of *S. aureus* skin and soft tissue infections occurred in patients who lived in the same area in different households and who were not related to the family clusters. With the exception of a case caused by CA-MRSA/ST8 in an infant 3 years of age (*22*), the other 10 cases were caused by MSSA. Nine patients were children ranging in age from 6 months to 11 years. Two infants (Table 1, patients 20 and 23) had experienced a pustular rash 1 year earlier, soon after birth in the Codogno Hospital. Clinical signs included pustulosis or furunculosis (3 patients) and abscesses (7 patients). One patient also had conjunctivitis. Patients were neither immunosuppressed nor had preexisting skin infections or other risk factors.

Antimicrobial drugs, mostly oral amoxicillin-clavulanic acid, were given to patients based on their clinical conditions and site and size of the infected area (*23*) (Table 1). A recurrence of a large subcutaneous abscess in 1 adult patient led to treatment with intravenous teicoplanin. Spontaneous drainage occurred in 4 abscesses, and surgical incision and drainage were performed in 3 cases. All patients had a favorable outcome.

Molecular Typing of Isolates from Community Infections

Isolates available for molecular typing included 5 MSSA from family clusters 2, 3, and 4, and 10 MSSA from the sporadic cases in 2006. All isolates from the family clusters and 7 of the 10 isolates from the sporadic cases contained PVL genes. By PFGE, all PVL-positive isolates appeared indistinguishable or closely related (differing by 1–2 bands) and were assigned to PFGE type A. These isolates also exhibited an identical *spa* type, corresponding to t005. MLST of 6 representative isolates yielded sequence type (ST) 22 (Table 1). Isolates with these characteristics will be subsequently referred to as the "epidemic MSSA clone." Three PVL-negative MSSA isolates, obtained from infections in 2006, showed different PFGE, *spa*, and MLST types (Table 1).

Skin and Soft Tissue Infections in Mothers and Neonates

Examination of medical records and microbiologic data from the Codogno Hospital showed a cluster of postpartum mastitis involving 13 women that had occurred from October 2003 through January 2004, before the family clusters were identified. The women had delivered in the same hospital 2–12 weeks before the onset of symptoms. In 6 case-patients, mastitis had progressed to breast abscesses and required surgical drainage. Culture of the drainage yielded MRSA in 1 case-patient and MSSA in the other case-patients. MSSA isolates had a susceptibility pattern identical to that of the community-acquired MSSA. Molecular studies were not performed on these isolates.

In early 2004, several cases of skin infections (mainly pustulosis of the groin or upper thigh) were observed in neonates in the hospital nursery or after discharge when they were examined during routine follow-up visits. From January through March 2004, 14 such skin infections were observed. No other cases were identified until December 2004, when 9 cases occurred. From January through September 2005, skin infections developed in a total of 65 neonates, with peak incidence in June and July when 14 and 17 cases were identified, respectively. In July 2005, screening for nasal *S. aureus* carriage was initiated, and infection control measures were enforced (see Infection Control Measures). Cases of infection gradually diminished, and no new cases were observed after September 2005. MSSA isolates were obtained from all neonates whose specimens had been cultured with skin infections in 2004 and 2005, but the isolates were not stored and were not available for molecular studies.

Screening for *S. aureus* Carriage and Molecular Typing of Carriage MSSA Isolates

To ascertain the circulation of *S. aureus* in the Codogno Hospital, screening for *S. aureus* nasal carriage was performed in the maternity ward and the nursery in July 2005 and in December 2005, after implementation of infection control measures. In July 2005, nasal swabs were obtained from 48 neonates, 58 mothers, and 71 medical and nonmedical personnel. MSSA was isolated from 19 (39.6%) of 48 neonates in the nursery, 16 (27.6%) of 58 mothers, and 19 (26.8%) of 71 staff (Table 2). No MRSA strain was identified. Remarkably, all of the 17 available isolates from neonates were PVL-positive and 16 corresponded to the epidemic MSSA clone. Only 1 maternal and 3 staff isolates corresponded to the epidemic MSSA clone. Personnel colonized with the epidemic MSSA included 1 pediatrician, 1 newborn nurse, and 1 nurse in the Department of Obstetrics and Gynecology. One of the neonates was colonized by a PVL-positive MSSA that had a PFGE profile, *spa* type,

Table 2. Results of nasal carriage screenings and molecular typing of PVL-positive MSSA isolates, northern Italy, 2005*

Persons sampled (no.)	MSSA carriers, no. (%)	MSSA isolates		PVL-positive isolates		
		No. examined	No. PVL+	PFGE type (no. isolates)	spa type	ST
July						
Neonates (48)	19 (39.6)	17	17	A (16)	t005	22
				B (1)	t021	956
Mothers (55)	16 (27.6)	10	1	A (1)	t005	22
Staff (71)	19 (26.8)	17	4	A (3)	t005	22
				A (1)	t2336	954
December						
Neonates (43)	0	0	0			
Mothers (17)	5 (29.4)	3	1	A (1)	t005	22
Staff (64)	3 (4.7)	3	2	D (1)	t645	1210
				E (1)	t1445	1209

*PVL, Panton-Valentine leukocidin; MSSA, methicillin-susceptible *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; ST, sequence type.

and MLST that were completely different from those of the epidemic MSSA. MSSA isolates that were PVL-negative were genotypically heterogeneous, showing a number of different PFGE profiles and *spa* types (data not shown).

In the December 2005 screening, nasal swabs were obtained from 43 neonates, 17 mothers, and 64 staff. No neonate was colonized with *S. aureus*, although 5 (29.4%) of 17 mothers and 4 (6.2%) of 64 staff members carried MSSA. In addition, a staff member was colonized with MRSA. Only 1 isolate from a mother corresponded to the epidemic PVL-positive MSSA clone, which suggests that although transmission inside the nursery had been interrupted, the epidemic strain was still circulating in the community. Two pediatric nurses were colonized by PVL-positive MSSA isolates that showed PFGE types, *spa* types, and STs that were unrelated to the outbreak MSSA clone (Table 2).

In all cases but 1, results of *spa* typing were in accordance with the PFGE analysis, by clustering the isolates belonging to the epidemic clone and discriminating genetically different isolates. The exception was a PVL-positive MSSA isolate obtained from a neonatal nurse in the July screening. This isolate was PFGE type A that had a novel *spa* type (t2336) resulting from deletion of 4 of the 12 repeats of t005, and yielded a novel combination of MLST alleles (ST954) that was a single locus variant of ST 22.

Infection Control Measures

When the neonatal outbreak of skin infections was identified in December 2004, contact precautions were instituted in the nursery and the maternity ward for staff and mothers, who were required to wear a gown and mask when feeding their babies. In June 2005, these control measures were expanded to include enhanced contact precautions based on existing recommendations to control the spread of MRSA and other drug-resistant microorganisms (*24*). Notices to promote handwashing among personnel as well as among mothers and visitors were posted on the walls of the nursery and waiting areas. The nursery and ad-

jacent areas were deeply cleaned with chlorine-containing disinfectant. After nasal carriage screening in July 2005, intranasal mupirocin was administered to all neonates and was continued until hospital discharge, usually for 5 days. Personnel and mothers with MSSA-positive nasal cultures received nasal mupirocin for 5 days with or without a 3-day course of amoxicillin/clavulanate. After the introduction of these measures, cases of MSSA skin infections in neonates decreased and no new cases occurred in the hospital after September 2005, no infections were reported at follow-up visits, and no neonates in the nursery carried *S. aureus* in the December 2005 nasal screening.

Discussion

We have described a large and prolonged outbreak caused by a PVL-positive MSSA strain that was probably initiated in the maternity ward and nursery of the local hospital and spread to the community. Striking similarities exist between the principal features of this outbreak and recent descriptions of outbreaks caused by typical CA-MRSA. First, skin infections occurred predominantly in children and young adults without risk factors, with intrafamilial spread and recurrences (several examples of familial transmission of CA-MRSA have been described in which family members can serve as a reservoir of CA-MRSA) (*25,26*). Second, the epidemic MSSA clone was prolonged in the community (in northern Denmark, a CA-MRSA strain was responsible for a community outbreak of recurring infections that involved 46 persons >6 years of age) (*27*). Third, a mastitis outbreak occurred and neonatal infections emerged in the local hospital (an outbreak of CA-MRSA infections in a neonatal intensive care unit was likely initiated by the mother of the index case who had a CA-MRSA wound infection and mastitis) (*28*). Twenty-one percent of neonates with CA-MRSA infections at the Texas Children's Hospital had a mother with a history of skin infections, including mastitis and axillary abscesses (*29*).

Our study has some clear limitations because the PVL-positive MSSA outbreak strain was only demonstrated in

isolates from 3 family clusters, in community infections in 2006, and in the hospital carriers. That the epidemic clone was responsible for the other 2 family clusters and the post-partum mastitis outbreak can only be inferred from the records of isolation of MSSA with a distinctive susceptibility pattern (i.e., resistant to penicillin and gentamicin only). As for infections in neonates, these were generally considered mild, and microbiologic cultures were performed in only a few cases. In addition to the microbiologic findings, epidemiologic and clinical data support the presence of an unusually virulent strain. In the Codogno Hospital outbreak, the temporal relationship between the mastitis outbreak and the emergence of neonatal MSSA infections suggests that the source of the strain might have been a mother who had undetected infection or colonization at delivery and transmitted the strain to the baby. Subsequently, the strain spread inside the newborn nursery, possibly with the contribution of colonized healthcare workers, leading to the colonization of babies and the emergence of skin infections a few days after birth. The colonized/infected mothers and neonates in turn spread the MSSA strain in the community.

Implementation of infection control measures, including enhanced hand hygiene, contact precautions, and mupirocin treatment, resulted in a rapid decline in the occurrence of neonatal infections and the disappearance of the strain among neonatal carriers. There are no established data to support prophylactic treatment with mupirocin in MSSA-colonized patients, although its use has been proposed for some colonized at-risk patients who will undergo surgery (30). Despite control in the hospital, skin infections caused by the epidemic MSSA clone continued in family clusters, and new cases unrelated to family clusters were recognized in 2006. In the family clusters, hygienic measures were suggested to avoid spread to other family members, but decolonization with mupirocin was not attempted. Two recent reports highlight the efficacy of mupirocin decolonization to terminate outbreaks of skin infections in the community (12,13).

Since June 2006, only 2 new cases caused by the epidemic MSSA strain were observed in 2 adult men in the community, 1 in 2007 and the other in 2008, indicating that the outbreak was controlled but that the strain had not disappeared from the community. In addition, in July 2007, the father in family cluster 1 experienced a recurrence of a chest abscess caused by the epidemic MSSA strain. Although no earlier isolate from that family was studied, the same epidemic strain was likely responsible for skin infections in that family over the span of at least 3 years.

The PVL-positive outbreak MSSA strain, characterized by t005 and ST22, is related to one of the major MRSA clones circulating in hospitals in the United Kingdom, where it has been designated EMRSA-15 (31). Although this clone, also known as ST22-IV, harbors the type IV staphylococcal cassette chromosome *mec* (SCC*mec*) and is uncommon in the United States (32), it is now emerging as a successful clone in several areas of the world (33,34). Characteristically EMRSA-15 is susceptible to gentamicin and resistant to erythromycin and ciprofloxacin (34).

Clonal group ST22 includes MRSA as well as MSSA (35), both of which can contain PVL genes. Recently, a PVL-positive ST22 MRSA strain caused a large outbreak in Bavaria (36). This strain was susceptible to most non–β-lactam antimicrobial agents, including erythromycin and ciprofloxacin. The MSSA strain responsible for the outbreak in northern Italy was susceptible to erythromycin and ciprofloxacin and resistant to gentamicin, resulting in a susceptibility profile highly divergent from that of EMRSA-15. The epidemic MSSA clone could represent the ancestor of a hospital MRSA clone or, less likely, a derivative of a hospital MRSA clone that emerged by deletion of SCC*mec*. Whichever is the case, PVL genes have been introduced into a genetic background associated with the ability to spread rapidly and cause epidemics.

The role of PVL in the pathogenesis of *S. aureus* infections is still controversial. Animal models of necrotizing pneumonia provide conflicting results (37,38). In a mouse model of skin infections, PVL did not seem to play an indispensable role (39). However, it is difficult to dismiss the simple observation that *S. aureus* isolates causing skin infections in humans are enriched for PVL (40). This is more striking in Europe, where PVL is associated with a variety of different *S. aureus* genotypes (5) and not with a single major clone as in the United States (2). Hypothetically, PVL could play a role that has not been explored in animal models, e.g., to enhance persistence in nasal colonization or survival on the skin. Alternatively, the PVL bacteriophage could confer other properties to *S. aureus* that contribute to the pathogenesis of skin infections.

The presence of PVL or the PVL bacteriophage may contribute to some of the characteristics of this clone that are shared with typical CA-MRSA, including its ability to persist in the human reservoir, to cause skin infections in healthy young persons, and to require enhanced infection control precautions in the hospital. The only distinctive difference with CA-MRSA infections is the wider spectrum of therapeutic options available that includes β-lactam antimicrobial agents. On the other hand, the hospital and community outbreaks were initially overlooked because the causative agent of the infections was an MSSA strain. This study underscores how the overall genetic background of the *S. aureus* strain and not the methicillin resistance trait per se, determines clinical severity and the epidemiologic features of infections.

Dr Tinelli is chief of the Division of Infectious and Tropical Diseases of the Hospital of Lodi, Italy. His main research interests are the surveillance and control of healthcare-associated infections, and HIV infection.

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Causes of Death in HIV-infected Persons Who Have Tuberculosis, Thailand

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Up to 50% of persons with HIV and a diagnosis of tuberculosis (TB) in Thailand die during TB treatment. In a prospective observational study, a team of physicians ascribed the cause of death after reviewing verbal autopsies (interviews of family members about events preceding death), laboratory data, and medical records. Of 849 HIV-infected TB patients enrolled, 142 (17%) died. The cause of death was TB for 38 (27%), including 6 with multidrug-resistant TB and 20 with disseminated TB; an HIV-associated condition other than TB for 50 (35%); and a condition unrelated to TB or HIV for 22 (15%). Twenty-three patients (16%) were judged not to have had TB at all. Death from all causes except those unrelated to TB or HIV was less common in persons receiving antiretroviral therapy (ART). In addition to increasing the use of ART, death rates may be reduced through expanded use of modern TB diagnostic techniques.

Tuberculosis (TB) is one of the most common causes of death among people living with HIV worldwide (1). In Southeast Asia, the death rate for HIV-infected TB patients during TB treatment is particularly high, ranging from 20%

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to 50% (2–5). HIV-infected patients in Southeast Asia are severely immunocompromised at the time of TB diagnosis, with a median CD4+ T-cell lymphocyte count (CD4) of 54–57 cells/μL (2,6–9). With this degree of immunosuppression, it is likely, but not known, that opportunistic infections other than TB contribute substantially to the high case-fatality rate.

Autopsy studies have helped delineate causes of death among people living with HIV, including HIV-infected TB patients, in sub-Saharan Africa (10–13). These studies found that the most common causes of death were TB, pneumonia, bacteremia, cerebral toxoplasmosis, and *Pneumocystis jirovecii* pneumonia (PCP). Autopsies are not routinely performed in HIV-infected persons in Asia, and data from Africa may not be generalizable to Asia. Malaria is much less common in Asia than in Africa, and HIV-infected TB patients have more severe immunosuppression and higher death rates than patients in Africa (2–4,6–9,14–19). Understanding actual causes of death may help with identifying effective interventions. In part, on the basis of autopsy studies of HIV-infected patients, programs began providing cotrimoxazole preventive therapy (CPT) to HIV-infected TB patients in Africa. This therapy protects against malaria, PCP, toxoplasmosis, and bacterial pathogens. The reported reduction in death rates from this intervention is large in Africa, but less so in Asia. (2,5,16,20–23). No published studies have demonstrated an association between CPT and reduced death rates in HIV-infected TB patients in Asia in the era of antiretroviral therapy (ART) (2,16; US Centers for Disease Control and Prevention [CDC], unpub. data).

Thailand has been greatly affected by the TB/HIV syndemic, i.e., 2 diseases acting synergistically to cause excess illness and death (24). Each year, TB develops in 91,000

persons, 15%–20% of whom are HIV-infected (*25,26*). To address the TB/HIV syndemic, Thailand recommends regular TB screening for persons with HIV, HIV testing for all TB patients, ART for all people with HIV who have a CD4 cell count <250, and CPT for all TB/HIV patients. Access to HIV treatment has been expanded nationwide. However, in practice, not all patients are treated according to the guidelines (*2*). To understand the causes of death in persons with HIV and a diagnosis of TB and identify possible interventions to reduce death rates, we conducted a prospective, multicenter, observational study of HIV-infected patients being treated for TB in Thailand.

Methods

Study Setting and Population

We conducted a cohort study of HIV-infected TB patients at the national infectious diseases referral hospital (Bamrasnaradura Institute) in Nonthaburi province and at public TB treatment facilities in Bangkok, Phuket, and Ubon-Ratchathani provinces. These facilities ranged from outpatient clinics to large public hospitals. There are no known, substantial differences in the HIV or TB epidemics across these 4 provinces. At all sites, treatment for HIV and TB were available from government providers using standardized government-recommended regimens. TB patients were eligible if they were HIV-infected, not pregnant, not incarcerated, ≥18 years of age, and receiving anti-TB treatment <4 weeks before study enrollment. Patients consenting to study enrollment were followed up from TB treatment initiation to the end of TB treatment. For this study, patients received the usual care for TB, HIV, and other diseases according to physician preference. We did not intervene to modify routine clinical practice. This study was approved by the ethical review committees of the Bangkok Metropolitan Administration, the Thailand Ministry of Public Health, and CDC.

Data Collection and Laboratory Studies

Patients had 3 study visits: at the beginning of TB treatment, at the end of the intensive phase of TB treatment (usually 2 months after start of treatment), and at the end of TB treatment (usually 6 months after treatment initiation). At the beginning of treatment, patients were interviewed using standardized study forms that asked about demographic characteristics, past and present medical history, knowledge and attitudes related to TB and HIV, and sexual behavior history and drug-use history. At every study visit, patients received a physical examination and provided information about medications taken and any adverse events experienced since their previous visit. Study staff reviewed medical records for any health-related problem that occurred between study visits.

At enrollment, blood samples were tested for liver function enzymes, viral hepatitis, complete blood count, and CD4 count. Sputum and specimens from extrapulmonary sites were collected for acid-fast bacilli smear and for mycobacterial culture, identification, and drug-susceptibility testing.

Although the standard TB treatment regimen in Thailand is 2 months of rifampicin, isoniazid, pyrazinimide, and ethambutol followed by 4 months of rifampin and isoniazid, some providers chose to use nonstandard regimens. Therefore, we categorized regimens used into those likely to be effective and those unlikely to be effective; regimens unlikely to be effective were those for which there were no clinical trials data or international guidelines to support use in HIV-infected TB patients (e.g., a 3-drug regimen of isoniazid, rifampin, and ethambutol) or those that may be appropriate for drug-susceptible TB but were prescribed for patients infected with drug-resistant strains. This classification was done by investigators who were blinded to the patient's treatment outcome.

Determination of Cause of Death

For patients who died during TB treatment, study staff obtained death certificates and medical records. Study staff also conducted a verbal autopsy for each patient. Verbal autopsies are a method for assessing causes of deaths, including those related to TB and HIV, in resource-limited countries (*27–32*). They involve interviewing a family member or friend who was closely associated with the patient during the period preceding death. These respondents were asked when the patient first became sick, what the symptoms were, how the patient died, and what the respondent believed was the cause of death. At enrollment, patients entering the study consented to allow a family member or friend to be interviewed in the event of their death. Likewise, the respondent also provided informed consent at the time of the interview. For patients who were lost to follow-up or transferred care to a facility not participating in the study, we reviewed the Thai government's vital status registry to determine whether they had died. Patients who died within 3 months and lost to follow-up or transfer were classified as deaths during study follow-up.

A committee of 3 physicians not involved in care of the patients reviewed the records of all deceased patients, including study forms, verbal autopsy reports, death certificates, medical records, and laboratory data. These 3 physicians reviewed cases together. A decision about the cause of death required agreement of 2 of the 3 physicians. After records review, the committee classified the cause of death as 1 of the following: TB, an HIV-related condition (not TB), or a non-TB/HIV–associated condition. The committee also recorded what it believed to be the specific cause(s) of death and its level of certainty about this determination:

uncertain, possible, probable, or highly certain. The level of certainty was based on 3 primary criteria: 1) laboratory, microbiologic, or pathologic evidence of the cause of death; 2) agreement between the physician panel and the cause of death as recorded on the death certificate or medical records; and 3) no other cause that was equally likely to have caused death. High certainty required all 3 of these criteria, probable required 2 of the 3, possible required 1, and uncertain was used for cases that lacked all 3 criteria. For some cases, the committee determined that the patient did not have TB. In these cases, there was no microbiologic evidence of TB, and the patient often had another condition that would explain the symptoms reported. Because TB programs would not have detected that patients undergoing treatment did not have TB, the patients therefore would still be registered. This retention is necessary because results should apply broadly to all HIV-infected persons registered for TB treatment.

Data Analysis

We described causes of death for all patients who died, stratified by level of certainty (highly certain and probable vs. possible and uncertain). We then compared causes of death for patients who died ≤60 days after TB diagnosis with those who died >60 days after TB diagnosis. Patients with evidence of disease in multiple sites were classified as having disseminated disease. TB localized to the central nervous system or abdomen was classified as complicated.

For univariate analysis of categorical variables, we compared proportions using the χ^2 test and, when appropriate, the Fisher exact test. For multivariate analyses of the association between medications used and specific causes of death, we performed a Cox proportional hazards multivariate analysis, after first confirming that the assumptions of the proportional hazards model were met. For this analysis, we excluded patients who died within 14 days after treatment initiation because we presumed these deaths were not preventable through medical treatment.

We created 4 separate models, 1 each for death caused by TB, death caused by an HIV-associated condition, death equally likely to be caused by TB or an HIV-associated condition, and death caused by a non-TB and non-HIV-associated condition. The outcome of interest was death due to the specific cause with any level of certainty (and excluding those who died of other causes), compared with patients who were alive. Outcomes other than death were censored after 1 year of follow-up.

We included data on use of CPT, ART, fluconazole, and an effective TB regimen, and we controlled for CD4. For CPT, ART, and fluconazole. Patients who had taken the medication for at least 14 days before their treatment outcome were categorized as being on the medication, whereas those not on the medication or on it for <14 days were cat-

egorized as not being on the medication. We also assessed for confounding according to hospitalization at enrollment, hepatitis C antibody reactivity, abnormal liver enzymes, and type of TB (pulmonary, extrapulmonary without complications, or disseminated/complicated extrapulmonary). We chose to assess for confounding among these variables because they were associated with risk for death in HIV-infected TB patients in Thailand (CDC, unpub. data). We developed our final models by using forward, step-wise variable selection, keeping variables with p<0.05 and those that modified the hazard ratios (HRs) by >10%.

Results

From May 1, 2005, through September 30, 2007, we enrolled 849 patients, of whom 142 (17%) died during TB treatment. Another 150 (18%) patients either were lost to follow-up or transferred their care to a facility not participating in the study. Among patients who died, the ascribed cause of death was TB for 38 (27%), an HIV-associated condition other than TB for 50 (35%), and a condition not related to TB or HIV for 22 (15%). TB or an HIV-associated condition was equally likely in 32 (23%) patients. Of the 142 patients who died, 23 (16%) were judged not to have had TB at all. Among the 74 patients for whom certainty about the cause of death was probable or highly certain, 29 (39%) died of TB, 33 (45%) died of an HIV-associated condition, and 12 (16%) died of a condition not related to TB or HIV (Table 1).

Of the 38 patients who died of TB, 20 (53%) had disseminated TB, including 3 who had disseminated multidrug-resistant (MDR) TB. Including those 3, a total of 6 (16%) had MDR TB. Seven patients had TB involving the central nervous system, including 2 with radiculomyelitis and 1 with MDR TB meningitis (Table 2).

Patients with HIV-associated deaths had a wide range of diagnoses. Among the 50 patients who died of an HIV-related cause other than TB, 10 (20%) died of nontuberculous mycobacterial infections, 7 (14%) died of PCP, and 8 (16%) died of other fungal infections (including 5 with cryptococcal meningitis) (Table 2). Of the 10 patients whose cause of death was determined to be nontuberculous mycobacteria (NTM), 4 had NTM isolated from a normally sterile site (2 from blood, 1 from bone marrow, 1 from a lymph node). NTM was isolated from sputum in 5 of the remaining patients and stool in the other.

A total of 32 patients died of a condition that was equally likely to be TB- or HIV-related, including 6 (19%) ascribed to disseminated mycobacterial disease. These diagnoses were based on multiple specimens being positive for acid-fast bacilli but no mycobacterial culture confirmation or identification. Finally, 22 patients died of a non-TB, non-HIV–associated condition, including 11 (50%) who died of liver disease (Table 2).

Table 1. Causes of death, stratified by level of certainty, for all enrolled patients who died, Thailand, 2005–2007*

Cause of death	Level of certainty, no. (%) patients		Total no. (%) patients, N = 142
	Probable or high, n = 74	Possible or uncertain, n = 68	
TB	29 (39)	9 (13)	38 (27)
HIV-associated condition (not TB)	33 (45)	17 (25)	50 (35)
TB or HIV-associated equally likely	0	32 (47)	32 (23)
Not TB or HIV-associated	12 (16)	10 (15)	22 (15)
*TB, tuberculosis.			

The distribution of causes of death varied when stratified by time from TB treatment initiation. When limited to the 74 patients for whom the cause of death was known with high or probable certainty, 18 (55%) of 33 deaths occurring <60 days after TB treatment initiation were caused by TB, compared with 11 (27%) of 41 deaths occurring >60 days after TB treatment initiation (p = 0.02). Of the 41 persons who died >60 days after initiating TB treatment, 23 (56%) died of an HIV-related condition, compared with 10 (30%) of the 33 patients who died <60 days after TB treatment initiation (p = 0.03).

The median CD4 for all patients enrolled was 55 (interquartile range [IQR] 18–142). Among patients who did not die, the median CD4 was 66 cells/µL (IQR 26–169). Median CD4 was 23 cells/µL (IQR 8.5–96) for persons who died of TB (p<0.01 for comparison with patients who did not die), 18 cells/µL (IQR 8–41) for those who died of an HIV-associated condition other than TB (p <0.01), 18 cells/µL (IQR 4–40) for those in whom TB and an HIV-associated cause of death other than TB were equally likely (p<0.01), and 63 cells/µL (IQR 18–112) among persons who died of a non-TB, non-HIV-associated cause (p = 0.18).

Use of ART, opportunistic infection prophylaxis, and an effective TB regimen, along with other characteristics had varying associations with death due to different causes. Of the 849 patients enrolled in the study, 371 (44%) received ART. Among the 142 patients who died, 36 (25%) received ART; 335/707 (47%) of persons not known to have died received ART. The risk for death caused by TB was lower for persons who took ART (HR 0.2, 95% confidence interval [CI], 0.1–0.5) and higher for patients who were prescribed an ineffective TB regimen (HR 5.0, 95% CI 2.0–12.6) and for those who were hospitalized at enrollment (HR 11.9, 95% CI 4.4–32.1). For death due to HIV-associated causes, ART was associated with decreased risk for death (HR 0.4, 95% CI 0.2–0.7), and being prescribed an ineffective TB regimen was associated with increased risk for death (HR 2.6, 95% CI 1.4–5.1). For patients in whom death due to TB or an HIV-associated cause was equally likely, the risk for death was lower for persons who were prescribed ART (HR 0.04, 95% CI 0.01–0.3) and fluconazole (HR 0.4, 95% CI 0.2–0.98). Decreased CD4 was associated with risk for death in all of these analyses, but use of CPT was not. ART, fluconazole, CPT, ineffective TB treatment, and CD4 were not associated with risk

for death from a non-TB, non–HIV-associated cause, but hepatitis C antibody reactivity and abnormal liver enzymes were associated with increased risk for death in this group (Table 3).

Discussion

Among HIV-infected persons with a TB diagnosis in Thailand, we found that TB-related deaths were most common within the first 2 months after initiation of TB treatment, but overall, >50% of all deaths occurring during TB treatment were not caused by TB, and some patients actually did not have TB. Multiple interventions are needed,

Table 2. Causes of death for all enrolled patients who died (N = 142), Thailand, 2005–2007*

Cause of death	No. (%) patients
TB	38 (27)
Disseminated TB (3 with MDR TB)	20 (53)
Central nervous system TB (1 with MDR TB)	7 (18)
Pulmonary TB (2 with MDR TB)	10 (26)
Peritoneal TB	1 (3)
HIV-associated condition	50 (35)
Bacterial infection	6 (12)
Cerebral toxoplasmosis	4 (8)
Disseminated CMV	1 (2)
Fungal infection (other than PCP)	8 (16)
Liver disease	1 (2)
Nontuberculous mycobacteria	10 (20)
PCP	7 (14)
Other infectious cause	5 (10)
Other noninfectious cause	1 (2)
Unknown	7 (14)
TB or HIV-associated condition equally likely	32 (23)
Disseminated mycobacterial disease (TB vs. NTM)	6 (19)
Liver disease	1 (3)
Other infectious cause	3 (9)
Other noninfectious cause	1 (3)
Unknown	21 (66)
Non-TB/HIV–associated condition	22 (15)
Bacterial infection	1 (5)
Liver disease	11 (50)
Stevens-Johnson syndrome	2 (9)
Other infectious cause	1 (5)
Other noninfectious cause	6 (27)
Unknown	1 (5)

*TB, tuberculosis; MDR TB, multidrug-resistant TB; CMV, cytomegalovirus; PCP, *Pneumocystis jirovecii* pneumonia; NTM, nontuberculous mycobacteria.

Table 3. Adjusted hazard ratios for associations between patient characteristics and causes of death among enrolled patients, Thailand, 2005–2007*

Patient characteristic	Death caused by TB, n = 723†	Death caused by non-TB HIV-associated cause, n = 745†	Death caused by TB or HIV equally likely, n = 726†	Death caused by non-TB/HIV condition, n = 719†
Used ART‡	0.2 (0.1–0.5)§	0.4 (0.2–0.7)§	0.04 (0.01–0.3)§	0.9 (0.3–2.6)
Used CPT‡	0.5 (0.1–1.5)	1.0 (0.3–3.2)	1.0 (0.3–3.5)	1.1 (0.2–5.5)
Used fluconazole‡	0.5 (0.2–1.2)	0.8 (0.4–1.6)	0.4 (0.2–0.98)§	1.2 (0.4–3.8)
CD4	0.993 (0.987–0.999)§	0.987 (0.980–0.994)§	0.988 (0.981–0.996)§	1.0 (0.996–1.004)
Ineffective TB regimen¶	5.0 (2.0–12.6)§	2.6 (1.4–5.1)§	0.3 (0.04–2.3)	0.9 (0.2–3.7)
Hepatitis C antibody positive	Not included	Not included	Not included	3.2 (1.2–8.3)§
Hospitalized at enrollment	11.9 (4.4–32.1)§	Not included	Not included	Not included
Abnormal liver enzyme levels#	Not included	Not included	Not included	5.3 (2.2–12.9)§

*TB, tuberculosis; ART, antiretroviral therapy; CPT, cotrimoxazole preventive therapy; CD4, CD4+ T-cell lymphocyte count; Not included, not retained in final model. Patient counts exclude 27 persons who died within 14 days of TB treatment initiation and 13 patients with missing CD4.
†Each model includes all patients who survived plus those who died of the specific cause noted (in each category, patients who died of any of the other 3 causes were excluded). Values in parentheses are 95% confidence intervals.
‡Must have been taken for ≥14 days to qualify as taking medication.
§$p<0.05$.
¶Ineffective regimens were those without supportive clinical trials data, without international guidelines, or not likely to work because of the drug-resistance pattern of the patient's isolate.
#Aspartate transaminase ≥120 units/L and/or alanine aminotransferase ≥165 units/L and/or bilirubin >2 mg/dL.

therefore, to reduce death rates in HIV-infected TB patients in Thailand.

Among patients who died of TB, delayed TB diagnosis may be partially responsible.. Of the 38 patients who died of TB, 30 had disseminated TB, MDR-TB, or complicated extrapulmonary TB, conditions that are difficult to diagnose, occur frequently in HIV-infected persons, and have high death rates (13,33,34). Hospitalization at enrollment was strongly associated with increased risk for death caused by TB but not death due to other causes, which further suggests that delay in TB diagnosis may be partially responsible. The World Health Organization recommends that countries with TB/HIV syndemics intensify TB case finding in HIV-infected persons and expand access to TB culture and drug-susceptibility testing (1,35). If implemented broadly, these strategies could reduce TB-related deaths by diagnosing TB before it is disseminated and severe and by allowing early initiation of second-line TB treatment for drug-resistant TB. Expansion in laboratory capacity and case finding will also need to be coupled to physician training. We found that use of regimens that are not standard or not tailored to the drug-susceptibility pattern of the TB strain was an important risk factor for death.

We also found that using ART during TB treatment was associated with reduced death rates both from TB and from non-TB, HIV-associated conditions. Previous epidemiologic studies in Thailand and other countries have demonstrated marked improvement in duration of survival among HIV-infected TB patients treated with ART during TB treatment (2,7,16,36–38). Our study confirms this finding and suggests that ART use most likely would dramatically reduce both early and late deaths. Clinical trials are currently attempting to identify the optimum time to initiate ART during TB treatment (39).

Among HIV-related causes of death other than TB, the most common causes were NTM disease and fungal infections. For the 4 patients who had NTM isolated from normally sterile sites, NTM most likely was a causative factor, but the role is less clear in those in whom it was isolated from sputum or stool. Expanding mycobacterial culture capacity will help better assess the impact of NTM disease in Asia because NTM may be an underappreciated cause of death among patients clinically diagnosed with TB (40). Fungal infections may be preventable with prophylactic antifungal treatment; a previous analysis of risk factors for death in Thailand found that fluconazole was associated with improved duration of survival (16). Controlled trials of antifungal prophylaxis may be needed to assess whether it increases survival rates among HIV-infected TB patients in Asia.

Although ART is associated with improved survival and fluconazole may be associated with reduced risk of some causes of death, CPT was not associated with reduced risk for death from any cause. This finding is consistent with that of several other observational studies from Southeast Asia in the era of ART (2,16; CDC, unpub. data). It is possible that the differing epidemiology of opportunistic infections in the region makes CPT less beneficial or not beneficial at all, or that these studies, none of which were randomized controlled trials, were not adequately controlled or powered to detect a meaningful difference. Randomized controlled trials of the efficacy of CPT in patients receiving ART may be needed in Southeast Asia.

In addition to these specific interventions, which could address the specific causes of death identified in this study, other interventions could decrease the high, early death rates observed in persons with HIV and a diagnoses of TB. First, the median CD4 count among all patients in this study was low. Earlier diagnosis of HIV through regular

provider-initiated testing and counseling of TB patients and earlier HIV testing of other persons combined with earlier initiation of ART would result in less immunocompromise and less risk for many of the opportunistic infections found in this population. Next, the impact of TB/HIV can be reduced by prevention of TB in persons with HIV (e.g., improved infection control measures in HIV care settings and use of isoniazid preventive therapy) and prevention of HIV in TB patients through appropriate counseling messages targeting persons with HIV and without HIV.

No method of assessing causes of death is completely reliable, particularly in resource-limited countries where microbiologic testing and postmortem examinations are infrequently performed and many patients die outside of hospitals. We used several imperfect data sources in combination—verbal autopsy, medical record review, death certificate data—to identify the cause of death. Both missed diagnoses and false diagnoses may have skewed our findings, but we could not determine the magnitude and impact of these problems without independent verification of the cause of death. Physicians determining cause of death could have been influenced by their own biases, but our use of a panel of physicians and criteria for ascribing causes should have limited this possibility. Finally, some providers did not always use available microbiologic tests, including blood culture. Failure to use these tests may result in underestimation of some causes of death.

We found that TB-related and HIV-related deaths are likely to be reduced through early initiation of ART and of appropriate anti-TB drug regimens. Expanded use of modern TB diagnostics may also improve survival by diagnosing TB before it is disseminated and severe, identifying drug resistance early, and differentiating between TB, NTM, and other causes of illness. Finally, improvements in general HIV care and treatment, including earlier HIV testing and ART use along with appropriate measures to prevent TB and HIV, should decrease the high early death rates observed.

Acknowledgments

Dr Cain is the team leader for TB/HIV in the International Research and Programs Branch of the Division of Tuberculosis Elimination at CDC. His main interests include TB and HIV in resource-limited settings and TB control among foreign-born persons in the United States.

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Medical Procedures and Risk for Sporadic Creutzfeldt-Jakob Disease, Japan, 1999–2008

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To elucidate the association between medical procedures and sporadic Creutzfeldt-Jakob disease (sCJD), we analyzed medical procedures (any surgical procedure, neurosurgery, ophthalmic surgery, and blood transfusion) for patients registered by the CJD Surveillance Committee in Japan during 1999–2008. We conducted an age-stratified case–control study with 753 sCJD patients and 210 controls and a study of patients who underwent neurosurgical or ophthalmic surgical procedures at the same hospital. Although the control group was relatively small, no evidence was found that prion disease was transmitted through the investigated medical procedures before onset of sCJD. After onset of sCJD, 4.5% of the sCJD patients underwent operations, including neurosurgical for 0.8% and ophthalmic for 1.9%; no special precautions against transmission of prion diseases were taken. Fortunately, we have not identified patients with prion disease attributed to these operations. Our findings indicate that surgical procedures or blood transfusion had little effect on the incidence of sCJD.

Prion disease is characterized by spongiform change and abnormal prion protein deposition in the brain and is transmissible under certain situations. Human prion disease is divided into 3 categories: genetic prion diseases with mutations of the prion protein (PrP) gene; prion diseases acquired by transmission of the prion through exposure to contaminated materials, including iatrogenic transmission; and sporadic Creutzfeldt-Jakob disease (sCJD) with no PrP mutation or evidence of exposure to prion. To date, >400 patients with iatrogenic CJD, who received prions through contaminated neurosurgical instruments, intracerebral electroencephalographic electrodes, human pituitary hormone, corneal transplants, or dura mater grafts, have been reported (*1*). Furthermore, some case–control studies reported that medical procedures were possible risk factors for sCJD (*2–6*). However, other studies did not demonstrate any significant association between medical procedures and sCJD (*7–10*).

After a results of a case–control study that found an association between CJD and medical procedures was reported from Japan in 1982 (*2*), 132 patients with dura mater graft–associated CJD (dCJD) have been found in Japan (*11,12*); however, no recent studies have investigated medical procedures as a risk for acquiring sCJD. In Japan, 66 (8.6%) of 766 patients with prion diseases had iatrogenic cases that were all dCJD (*12*), and the outbreak of iatrogenic CJD required a new study about the association between sCJD and medical procedures in Japan. Here we analyzed the role of medical procedures in cases of sCJD by using relevant data from CJD surveillance in Japan.

Methods

Patients

We investigated 1,339 patients with suspected prion diseases who had been registered by the CJD Surveillance Committee in Japan from April 1999 through February 2008. The surveillance system was initiated in April 1999, and each patient was prospectively assessed with a surveillance protocol that assembled information about life

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history; previous medical history, including the history of surgical treatment and blood transfusion; clinical history; laboratory data; and results of molecular genetic and pathologic examinations. Information on patients with suspected prion diseases were obtained through 1) the application for registration with the Japanese Intractable Diseases Information Center (www.nanbyou.or.jp/english/nan_kenkyu_45. htm) by each patient's family, 2) the law on infectious diseases, or 3) request for genetic or cerebrospinal fluid analyses sent to members of the CJD Surveillance Committee by the physicians. In Japan, 123 diseases have been defined as intractable disease, and for 45 of them, including prion diseases, patients receive additional economic support for medical costs. Furthermore, medical doctors must report patients suspected of having prion disease to the local public health department within 7 days after the diagnosis, according to the law on infectious diseases (which has been enforced since April 1999 in Japan to monitor some specific infectious diseases). After written consent approved by the Institutional Ethics Committee was obtained from each patient's family, members of the CJD Surveillance Committee directly examined the patient and collected data from the clinical records. For each patient with a history of surgery, we collected information about the underlying disease from the patient's family, including the date and hospital in which the operation was performed. For each patient with a history of blood transfusion, we collected information about the date of blood transfusion. Most information was collected by interviewing the patient's family members.

On the basis of discussions by the CJD Surveillance Committee, we confirmed or denied the diagnosis of prion disease in each case. In patients with a confirmed diagnosis of prion disease, we classified prion diseases into 4 categories: sCJD, acquired prion disease, genetic prion disease, and unclassified prion disease. sCJD was diagnosed according to the revised classical criteria established by Masters et al. (13): definite CJD (neuropathologically confirmed spongiform encephalopathy or abnormal prion protein deposition in the brain); and probable CJD (neuropathologically unconfirmed cases showing progressive dementia, periodic sharp-wave complexes on electroencephalogram, and at least 2 of the following features: myoclonus, pyramidal signs/extrapyramidal signs, cerebellar signs or visual symptoms, and akinetic mutism). Acquired prion diseases included iatrogenic CJD, in which the criteria for sCJD were applied for a diagnosis with a history of iatrogenic exposure, and variant CJD, in which the diagnosis was based on the World Health Organization (WHO) 2001 criteria (14). Regarding the accuracy of the diagnosis of genetic prion diseases, pathologically verified cases were defined as "definite," and cases demonstrating mutations in the PrP gene and neuropsychiatric manifes-

tations compatible with prion diseases were defined as "probable." We selected patients with definite or probable sCJD for analysis.

Patients who did not receive a diagnosis of prion diseases were classified into 3 categories: prion diseases definitely denied; prion diseases probably denied; and diagnosis unclear. "Prion diseases definitely denied" indicated patients whose conditions were definitively diagnosed as diseases other than prion diseases, and "prion diseases probably denied" indicated patients for whom the diagnosis of prion diseases was clearly unlikely due to the improving or nonprogressive disease course or for other reasons, although a definitive diagnosis of another disease was not established. Because patients with "prion diseases definitely denied" or "prion disease probably denied" had no or little possibility of prion disease, we selected these cases as the controls in our case–control study.

Surgical Procedures and Blood Transfusions before Onset of sCJD

To estimate the risk for sCJD through past surgery or blood transfusion, we performed a case–control study. Operations were divided into the following categories: neurosurgery, ophthalmic surgery, and surgery other than neurosurgery or ophthalmic surgery (other surgery), because neurosurgery or ophthalmic surgery for those with prion diseases are categorized in the guidelines of the CJD Incident Panel in the United Kingdom as high- or medium-risk procedures for transmission of infective PrP (15). In these guidelines, procedures involving the olfactory epithelium are also categorized as medium risk (15). However, the number of persons who underwent the operation possibly involving the olfactory epithelium is too small to be estimated by statistical analysis (2 sCJD patients and 2 controls underwent surgery for sinusitis), and we categorized these operations as other surgery. Neurosurgery included operations on the brain, cerebral blood vessels, and spinal cord. Ophthalmic surgery included all operations involving the eyeball and optic nerve. Other surgery included all surgical procedures other than neurosurgery and ophthalmic surgery. Furthermore, the committee performed a detailed investigation of sCJD patients who underwent neurosurgery or ophthalmic surgery at a hospital where other patients with any type of prion disease had ever undergone neurosurgery or ophthalmic surgery.

Surgical Procedures after Onset of sCJD

We analyzed sCJD patients who underwent surgical procedures after the onset of sCJD because such procedures might cause secondary transmission of the disease through contaminated instruments. In particular, for neurosurgery and ophthalmic surgery, we investigated the reason for the operation, interval between the operation and onset

of sCJD symptoms, age at onset of sCJD, and symptoms at onset of sCJD.

Statistical Analyses

Between the sCJD and control groups, age at onset was compared by Student *t* test, and medical procedures before the onset of diseases were compared by Fisher exact test. The case–control study of surgical procedures and blood transfusions before the onset of diseases was estimated by logistic-regression analysis. Because age at onset was different among sCJD patients (mean ± SD, 67.7 ± 9.5 years) and controls (59.3 ± 16.6 years) (p<0.0001), we divided the sCJD patients and controls into 3 categories according to age at disease onset; 31–50 years, 51–70 years, and ≥71 years. We performed a single regression analysis for any operation, neurosurgical procedure, ophthalmic surgical procedure, other operation, and blood transfusion in each age group. The strength of association between sCJD and putative risk factors was assessed by the odds ratios and 95% confidence intervals. Significance was defined as p<0.05. Statistical analyses were performed by using StatView J-7.5 (Abacus Concepts, Berkeley, CA, USA).

Results

A total of 990 patients received a diagnosis of definite or probable prion disease. Summary of the characteristics of patients with prion diseases is shown in Table 1, in which 760 patients with sCJD are included. There were 221 patients with "prion disease definitely denied" and "prion disease probably denied." Seven sCJD patients and 11 control patients were excluded from the case–control study because information on medical history was not sufficient for analysis. Diagnoses of the 210 control patients is shown in Table 2.

Medical Procedures before Onset of sCJD

Frequencies of medical procedures before the onset of sCJD in sCJD patients and in controls are compared in Table 3. For both the sCJD and control groups, ≈50% had a history of surgery, and ≈10% had received a blood transfusion. No significant differences were found between them in frequency of any surgery, neurosurgery, ophthalmic surgery, other surgery, or blood transfusion (Table 3). In the logistic-regression analysis, no significant risk was associated with any medical procedures investigated in this study (Table 4).

Five sCJD patients had a history of neurosurgery or ophthalmic surgery at hospitals where neurosurgery or ophthalmic surgery had been performed on patients in whom prion disease later developed (Table 5); intervals between operations at the same hospitals were >3 years (Table 5).

Table 1. Characteristics of patients with definite or probable prion disease, Japan, 1999–2008*

Type of prion disease	No. (%) patients
Sporadic CJD	760 (76.8)
Genetic prion diseases	167 (16.9)
Acquired prion diseases†	62 (6.3)
Unclassified CJD	1 (0.1)
Total	990

*CJD, Creutzfeldt-Jakob disease.
†Acquired prion diseases included 61 cases of dura mater CJD and 1 case of variant CJD.

Surgical Procedures after Onset of sCJD

Except for 2 patients suspected of having prion disease, who had undergone brain biopsy with disposable instruments, 34 (4.5%) of 760 sCJD patients underwent some type of surgical procedure before the diagnosis of prion disease, including neurosurgery in 6 (0.8%), ophthalmic surgery in 14 (1.8%), and other surgery in 16 (2.1%). The 6 case-patients who underwent neurosurgery had these operations within 3 months after sCJD onset: procedures performed for subdural hematoma (n = 3), aneurysm (n = 2), and menigioma (n = 1) (Table 6). All 14 case-patients who underwent ophthalmic surgery underwent operations for cataracts, and 7 of these patients had had visual disturbance as an initial symptom of sCJD (Table 7). Among 5 patients for whom information on the effects of ophthalmic surgery could be obtained, 2 had some improvement of visual symptoms after surgery, but the other 3 patients had no improvement. Although both cataracts and sCJD could contribute to the visual symptoms, sCJD would contribute to visual symptoms in patients who had no effects of ophthalmic surgery. We have obtained information about instrument cleaning and sterilization procedures for 3 of 5 patients who underwent neurosurgery and for 5 of 14 patients who underwent ophthalmic surgery after the onset

Table 2. Diagnoses for 210 controls in case–control study of sCJD, Japan, 1999–2008*

Disease	No. diagnoses
Encephalitis	27
Alzheimer disease	21
Frontotemporal dementia	15
Metabolic encephalopathy	15
Cerebrovascular disorders	12
Spinocerebellar degeneration	12
Corticobasal degeneration	9
Epilepsy	7
Psychiatric disorders	7
Hypoxic encephalopathy	7
Hashimoto encephalopathy	6
Dementia with Lewy bodies	6
Paraneoplastic syndrome	5
Mitochondrial encephalopathy	4
Malignant lymphoma	3
Other disorders	54

*sCJD, sporadic Creutzfeldt-Jakob disease.

Table 3. Medical procedures before disease onset, case–control study of sCJD, Japan, 1999–2008*

Medical procedures	sCJD case-patients, no. (%), n = 753	Controls, no. (%), n = 210
Surgery	372 (49.4)	104 (49.5)
Neurologic	25 (3.3)	13 (6.2)
Ophthalmic	42 (5.6)	11 (5.2)
Other	337 (44.8)	89 (42.4)
Blood transfusion	78 (10.4)	20 (9.5)
*sCJD, sporadic Creutzfeldt-Jakob disease. p values were not significant.		

of sCJD. All surgeons reused some of the surgical instruments, but according to the WHO guidelines (*16*), the sterilization methods of the instruments were not appropriate for eliminating infectious PrP, including the use of ethylene oxide gas or incomplete autoclaving.

Discussion

In this case–control study, we found no evidence of increased sCJD risk associated with patient's history of surgical procedures or blood transfusions. In the previous case–control study and in our study, receipt of a blood transfusion was not shown to be a significant risk for CJD (*2–10*). However, whether surgical procedures contribute to the risk for sCJD has been controversial. Our results, in which any operation was not a significant risk for sCJD, were consistent with results of 2 previous large case–control studies (*8,9*) and a reanalysis of results of 3 case–control studies (*10*). Even in the studies with positive results, some different results were provided when the surgical procedures were categorized by affected organ. One previous case–control study indicated significant risk for sCJD after neurosurgical procedures (*3*), but no significant risk was shown in other studies (*5,6,8–10*). Ophthalmic surgery was reported as causing significant risk for sCJD in a case–control study in Australia (*4*) but not in other studies (*5,6–10*).

In a recent study in the United Kingdom (*6*), the increased risk associated with having undergone surgical procedures was restricted to the category "other surgery," which included such procedures as sutures to skin, and the association largely disappeared when the whole of the other-surgery category was excluded. These different results may show little possibility for transmission of infectious PrP through surgical procedures, although we cannot exclude the possibility that such transmission occurs occasionally because iatrogenic CJD exists.

The conflicting results in case–control studies, including ours, may be explained by differences in the area, race, period in which studies were performed, number of patients, and methods as discussed below. Our study, which attempted to determine when medical procedures were associated with an increased risk for sCJD, had the largest number of sCJD patients in case–control studies to date. The relatively small number of controls is a potential limitation. In case–control studies, methods of obtaining data from controls should be the same as those from patients. In our study, patients in the groups "prion diseases definitely denied" or "prion diseases probably denied" in our CJD surveillance, who had no or little possibility of having prion disease, were used as the controls. Therefore, data from controls could be collected at the same level of precision as those from the sCJD cases. Because the ages of the sCJD patients and controls were significantly different, age-stratified analysis was required in our study. A recent study reported that some methodologic differences might partially explain conflicting data regarding the association between surgical procedures and CJD (*17*). The report suggested that the use of controls from the community would be preferable to using those from the hospital because community-based controls are often more representative and would result in a more valid comparison (*17*). Furthermore,

Table 4. Medical procedures and risk for sCJD, by age at disease onset, Japan, 1999–2008*

Age range, y	Data category	Total no. patients	Any surgery	Neurosurgery	Ophthalmic surgery	Other surgery	Blood transfusion
31–50	sCJD	32	50.0%	6.3%	6.3%	40.6%	3.1%
	Control	37	45.9%	10.8%	2.7%	37.8%	5.4%
	OR		1.66	0.38	2.15	0.78	0.64
	95% CI		0.04–74.09	0.02–6.64	0.05–101.51	0.02 – 33.39	0.05–9.09
	p value		0.79	0.50	0.70	0.90	0.74
51–70	sCJD	414	43.7%	1.7%	2.2%	41.8%	9.4%
	Control	97	46.4%	5.2%	3.1%	40.2%	11.3%
	OR		0.18	0.69	2.71	5.57	0.84
	95% CI		0.02–1.73	0.13–3.62	0.24–30.38	0.62–50.05	0.40–1.77
	p value		0.14	0.66	0.42	0.13	0.64
≥71	sCJD	317	57.0%	5.2%	10.1%	49.2%	12.4%
	Control	60	65.0%	6.7%	10.0%	56.7%	11.7%
	OR		0.81	0.76	1.15	0.83	1.27
	95% CI		0.15–4.37	0.15–3.80	0.38–3.48	0.17–4.02	0.52–3.10
	p value		0.80	0.74	0.81	0.82	0.60
*sCJD, sporadic Creutzfeldt-Jakob disease; OR, odds ratio; CI, confidence interval.							

Table 5. Characteristics of 5 sCJD patients who underwent neurosurgery or ophthalmic surgery at hospitals where other patients with prion diseases had previously undergone neurosurgery or ophthalmic surgery, Japan, 1999–2008*

Patient	Type of CJD	Onset of CJD	Date of surgery	Reason for surgery
1	sCJD	2003 Aug	1991 Aug	Subarachinoid hemorrhage
	dCJD	2001 May	1976	Spinal cord tumor
			1986 Aug	Spinal cord tumor
2	sCJD	2002 Feb	1994 Sep	Subdural hematoma
			1997 Sep	Cataract
	dCJD	1998 Jan	1987 Jan	Meningioma
3	sCJD	2001 Jan	1989 Apr	Subarachnoid hemorrhage
	dCJD	1995 Jul	1980 Jul	Aneurysm
4	sCJD	2001 Jul	1999	Spinal cord lesion (details unknown)
	dCJD	2001 Aug	1978 Sep	Astrocytoma
5	sCJD	2002 May	2002 Apr	Cataract
	sCJD	2002 May	1997 Aug	Cataract
			1999 Jan	Cataract

*CJD, Creutzfeldt-Jakob disease; sCJD, sporadic CJD; dCJD, Creutzfeldt-Jakob disease associated with cadaveric dura mater graft.

using proxy informants for controls may be advisable for the purpose of comparability with case-patients, although this practice does not necessarily offset biases in data ascertainment (*17*). In our case–control study, we used proxy informants for controls who were recruited from hospitals under the same condition as the sCJD case-patients.

Regarding the 5 sCJD patients with a history of neurosurgical or ophthalmic surgical procedures at hospitals where other patients with prion disease had previously undergone such procedures, we consider that the possibility of transmission through these procedures was extremely limited because the intervals between procedures and the acquisition of sCJD had been >3 years for all patients. According to the Incident Panel in the United Kingdom, most instruments that have gone through 10 cycles of use and decontamination are unlikely to pose a substantial risk (*15*). We assume that all instruments had gone through >10 cycles of use during the 3-year interval, and almost no infectivity remained on the instruments. In Japan, a large number of dCJD patients have been recognized with no other types of iatrogenic CJD (*11,12*); this study confirmed that no surgically transmitted cases occurred among patients with sCJD.

It is noteworthy that 4.5% of the sCJD patients underwent some types of surgical procedures after the disease onset, including neurosurgical (0.8%) and ophthalmic procedures (1.8%). Through surgical instruments, neurosurgi-

cal operations may transmit high infectivity from the brain tissues of sCJD patients, and ophthalmic operations may transmit moderate infectivity of the eye tissues in cases of cataracts (*15*). In this study, all these neurosurgical and ophthalmic procedures were performed without suspicion of prion diseases or special precautions to reduce the risk for secondary transmission of prion infection through the instruments. These findings suggest that delayed diagnosis of sCJD would be linked to increased risk for secondary transmission of prion diseases through surgical instruments. In neurosurgical procedures, the symptoms of sCJD were misdiagnosed as those of other neurologic diseases, and operations were performed near the time of disease onset. In terms of ophthalmic surgery, all patients underwent operations for cataracts, and 7 (50%) of 14 patients had visual disturbances as an initial symptom of sCJD. These data are similar to those in a report from the United Kingdom (*18*). Visual disturbances might prompt ophthalmic surgery. More seriously, 3 patients underwent operations ≥8 months after sCJD onset. In this study, all surgeons who provided information reused the surgical instruments with incomplete sterilization, and the potential for infection was the same as in our previous study of ophthalmic surgery (*19*).

Neurosurgeons and ophthalmologists should become better informed about prion diseases and the necessity of using disposable instruments whenever possible. Further-

Table 6. Data for sCJD patients who underwent neurosurgery after onset of sCJD symptoms, Japan, 1999–2008*

Patient no.	Reason for surgery	Interval between onset of sCJD symptoms and surgery, mo	Age at onset of sCJD, y	Symptom at onset of sCJD
1	Subdural hematoma	0	71	Dementia
2	Subdural hematoma	0	77	Apathy
3	Subdural hematoma	1	57	Dementia
4	Meningioma	1	74	Vertigo
5	Aneurysm	2	46	Dementia
6	Aneurysm	3	67	Vertigo

*sCJD, sporadic Creutzfeldt-Jakob disease.

Table 7. Data for sCJD patients who had ophthalmic surgery for cataracts after onset of sCJD symptoms, Japan, 1999–2008*

Patient no.	Interval between onset of sCJD symptoms and surgery, mo	Age at onset of sCJD, y	Symptom at onset of sCJD
1	0	60	Gait disturbance
2	0	61	Dementia
3	0	63	Visual impairment
4	0	71	Visual impairment
5	0	74	Visual impairment
6	0	74	Visual impairment
7	1	66	Dementia
8	1	74	Depression
9	1	85	Visual impairment
10	2	79	Tremor
11	4	81	Visual impairment
12	8	77	Anxiety
13	10	57	Dementia
14	14	64	Visual impairment

*sCJD, sporadic Creutzfeldt-Jakob disease.

more, a more sensitive method for early diagnosis of sCJD is needed because clinical diagnosis is sometimes difficult, particularly in atypical sCJD cases, such as MM2, MV2, VV1, or VV2 types (20–23), according to 6 phenotypes of sCJD divided by codon 129 polymorphisms of PrP (methionine/valine) and type of infectious PrP by Western blotting (24). Even neurologists may misdiagnose the initial stage of the atypical sCJD cases as being another neurodegenrative disease such as Alzheimer disease and progressive supranuclear palsy (20). Moreover, patients who have undergone surgical procedures with possibly contaminated instruments need to undergo a risk assessment with long-term follow-up after careful ethical consideration. Since June 2004, we have identified and monitored all patients who underwent neurosurgical procedures with possibly contaminated instruments, CJD has developed in none of those patients.

In conclusion, we did not demonstrate any evidence of increased risk for sCJD associated with a history of surgery or blood transfusion in the Japanese surveillance system. However, the fact that some patients had surgeries, including neurosurgery, even after the onset of sCJD indicates that we cannot deny any possibility of transmission of prion diseases by medical procedures. Neurosurgeons, ophthalmologists, and other surgeons need to focus more attention on prion diseases to reduce the iatrogenic risk, as well as realize that prolonged, careful surveillance of prion diseases is necessary.

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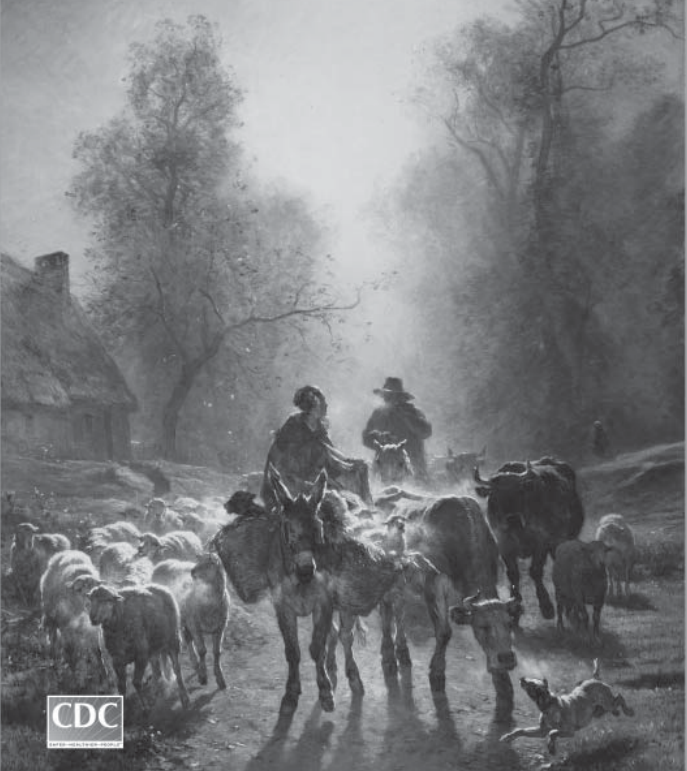
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Highly Pathogenic Avian Influenza Virus (H5N1) in Frozen Duck Carcasses, Germany, 2007

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We conducted phylogenetic and epidemiologic analyses to determine sources of outbreaks of highly pathogenic avian influenza virus (HPAIV), subtype H5N1, in poultry holdings in 2007 in Germany, and a suspected incursion of HPAIV into the food chain through contaminated deep-frozen duck carcasses. In summer 2007, HPAIV (H5N1) outbreaks in 3 poultry holdings in Germany were temporally, spatially, and phylogenetically linked to outbreaks in wild aquatic birds. Detection of HPAIV (H5N1) in frozen duck carcass samples of retained slaughter batches of 1 farm indicated that silent infection had occurred for some time before the incidental detection. Phylogenetic analysis established a direct epidemiologic link between HPAIV isolated from duck meat and strains isolated from 3 further outbreaks in December 2007 in backyard chickens that had access to uncooked offal from commercial deep-frozen duck carcasses. Measures that will prevent such undetected introduction of HPAIV (H5N1) into the food chain are urgently required.

Highly pathogenic avian influenza (HPAI) causes a substantial proportion of deaths in susceptible poultry species, which potentially lead to severe economic losses

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(*1*). Some HPAI viruses (HPAIV), in particular HPAIV of subtype H5N1 of Asian origin (*2*), exhibit a zoonotic potential, causing rare, but often fatal disease, in humans.

Since this virus was first detected in southern People's Republic of China in 1996, descendants of this virus have spread among poultry in 3 continents; as yet, only the Americas and Australia have been avoided. In an unprecedented scenario, wild birds have also been widely affected by HPAIV (H5N1) and are believed to have contributed to its transcontinental spread (*3*). In central Europe, HPAIV (H5N1) infections were encountered for the first time in winter 2005–06 and spring 2006, when mainly wild birds, but also a few isolated poultry holdings, were affected. Since August 2006, the virus seemed to have disappeared, but it reemerged in January and February 2007 in Hungary and the United Kingdom (*4*). All of these outbreaks were attributable to virus strains of phyloclusters 2.2, groups A and B, which originated from unknown sources but had phylogenetic links to viruses isolated during outbreaks of HPAI among wild birds at Lake Qinghai in northwestern China in 2005 (*4,5–8*).

In July and August 2007, introduction of yet another Qinghai-like subcluster of subtype H5N1 viruses, designated 2.2 group C, led to additional cases, first in poultry in the Czech Republic and later in wild birds in France and Germany. In Germany, outbreaks also occurred in several poultry holdings, including 2 large duck-fattening farms. However, the infected ducks did not show clinical symptoms indicative of avian influenza, and no overt excess daily deaths were observed in these holdings. Outbreaks in poultry were spatially and temporally linked to cases in wild birds. Control measures included the culling of >750,000 animals (*9*). These measures seemed to contain the outbreaks because no more cases became apparent after August 2007. Phylogenetic studies indicated

that these outbreaks had a common, as yet unidentified, source (*7*).

In December 2007, HPAIV (H5N1) was detected in 3 isolated backyard holdings in the Federal State of Brandenburg in northeastern Germany, although no concomitant cases of subtype H5N1 infection had been detected in wild birds or in poultry since August 2007. This puzzling situation prompted detailed field epidemiologic investigations. These investigations, corroborated by results from wild bird monitoring and from phylogenetic analysis of the respective viruses, indicate that wild birds can be ruled out with high reliability as a source of infection in these cases. Instead, infected duck meat, possibly originating from the German duck-fattening farms affected by the outbreaks in August 2007, might have caused these cases.

Materials and Methods

Detection of Virus

RNA from swab samples or tissues was isolated by manual (Viral RNA Kit; QIAGEN, Hilden, Germany, and Tecan Evo 3000 System; Macherey-Nagel, Düren, Germany) procedures. One-step real-time reverse transcription–PCR (rRT-PCR), which specifically amplified fragments of the avian influenza virus (AIV) M, H5, H7, or N1 genes, was performed as reported in European Commission (EC) decision 2006/437/EC. Pathogenicity assessments were based on molecular analysis of the H5 cleavage site by either rRT-PCR (*10*) or sequencing (*11*). Virus isolation was performed in the amnioallantoic cavity of 9- to 11-day-old embryonated hens' eggs.

Nucleotide Sequencing of Virus Genes

RNA was prepared from allantoic fluid of inoculated embryonated hens' eggs. Generation of full-length gene amplification products of the hemagglutinin (HA) gene and sequencing were performed as previously described (*7*). Sequences are publicly available from GenBank: R1359/07-AM 749 443; R1349/07-AM 749 442; R1393/07-AM 773 724; R1400, 1406, 1772, 1779, 1797, 2048/07-AM 914 004/012/014/016/021/026; R3234, 3272, 3294/07-FM 177 119/127/135; and R3248, 3249/07-FM 163 440/448.

Phylogenetic Analyses

Sequences of full-length HA genes were aligned by using the multiple sequence comparison by log-expectation (MUSCLE) method (www.ebi.ac.uk/Tools/muscle/index.html) and were then subjected to distance matrix calculations (FastME; *12*). Minimal-evolution (ME) trees were built by using the default options of FastME (balanced greedy minimal evolution to build the initial tree and balanced nearest-neighbor interchanges for swapping

and optimization). In addition, maximum-likelihood (ML) analysis (TreePuzzle; *13*) was performed by using the public Phylemon server (http://phylemon.bioinfo.cipf.es/cgi-bin/tools.cgi). One thousand (ME) or 200 (ML) bootstrapping cycles were performed.

Serologic Testing

Poultry serum specimens from affected and in-contact holdings were screened by using commercially available competitive ELISA (cEIA) kits that detect antibodies specific for the nucleocapsid protein (Pourquier AI A Blocking ELISA; Institut Pourquier, Montpellier, France, or ID Screen Influenza A NP Antibody Competition ELISA; ID VET, Montpellier, France) according to the manufacturers' instructions. Internal validation data showed that performance characteristics of these 2 assays were largely comparable with those of duck sera. Consequently, they were used interchangeably in the different laboratories. Positive serum specimens were further analyzed by hemagglutination inhibition (HI) assay according to 2006/437/EC. Antigens prepared from subtypes H5N2 (A/ostrich/Denmark/72420/96; Veterinary Laboratories Agency [VLA], Weybridge, UK), H5N1 (NIBRG14, NIBSc, UK), and H7N7 (A/tk/England/647/77; VLA) viruses were used.

Results

Outbreak Detection and Field Epidemiologic Investigations

Bavaria

In an industrial duck-fattening farm (farm A) in Bavaria, Germany, a slight increase of daily mortality rates ranging from 0.7% to 1.8% from August 19, 2007, onward was registered in barn A/15, which prompted swab sampling on August 22, 2007 (Table). Although initially *Riemerella* spp. were detected, differential diagnostic measures included PCRs for AIV (H5N1), which yielded positive results. HPAIV (H5N1) was finally confirmed on August 25, 2007, which led to the culling of all 170,000 ducks kept at that time at farm A. Further sampling at culling led to detection of HPAIV (H5N1) in 3 other barns of farm A (nos. 10, 12, and 13; Table).

Farm A also operated a large regional poultry abattoir. Thus, a considerable number of contact farms, most of them keeping ducks for fattening, including farms B and C, were identified. Except for farms B and C, no clinical, virologic, or serologic evidence for spread of virus was obtained in monitoring investigations. Farms B and C were serviced by the same crew of poultry workers and, hence, were treated as a single epidemiologic unit. No clinical evidence for an HPAIV infection was obtained on August 28, 2007, and

Table. Summary of investigations for HPAIV (H5N1) infections in industrial duck-fattening farms A, B, and C by rRT-PCR, sequencing and serologic analyses, Germany, 2007*

Farm/barn unit	Date of housing	Herd size	Date of culling	Cumulative proportion of deaths, %	Duckling age, d	Swab samples†	Serum samples‡	Tissue samples†
A/10	13 Jul	12,015	25 Aug	14.4§	43	50/6 (25 Aug)	30/30/6	–
A/12	16 Jul	39,165	26 Aug	12.5	41	50/39 (25 Aug)	30/13/1	–
A/13	13 Jul	14,000	25 Aug	14.4§	43	50/23 (25 Aug)	30/30/4	–
A/15	1 Aug	45,696	25 Aug	10.2	25	25/13 (22 Aug)	–	–
B/1-1	29 Jun	32,540	11 Aug¶	20.3 (Figure 1, panel C)	43	–	–	7/(1)
B/1-1	14 Aug	35,175	9 Aug	ND	24	519/0 (5 Sep)	106/5/0	–
B/1-2	17 Aug	35,000	9 Aug	ND	21	511/0 (5 Sep)	109/5/0	–
B/2-4	20 Jul	35,860	9 Sep	5.9 (Figure 1, panel D)	50	511/6# (5 Sep)	126/105/52	122/1
B/3-5	25 Apr	22,550	14 Jun¶	12.6 (Figure 1, panel A)	50	–	–	–
B/3-5	20 Jun	36,300	8 Jan¶	8.3 (Figure 1, panel B)	42	–	–	34/2
B/3-5	7 Aug	35,860	9 Oct	2.4	34	515/0 (5 Sep)	125/3/0	–
B/3-6	10 Aug	34,650	9 Oct	1.2	31	519/0 (5 Sep)	130/1/0	–
C	19 Jul	28,000	9 Oct	2.2	52	515/0 (5 Sep)	91/3/0	–

*HPAIV, highly pathogenic avian influenza; rRT-PCR, real-time reverse transcription–PCR; ND, no data available.
†Total number of samples (oropharyngeal swabs; lung and central nervous system tissues) examined by rRT-PCR and sequencing/no. of subtype H5N1-positive samples; date of swab collection is indicated in parentheses.
‡Total number of serum samples examined/nos. positive for nucleocapsid protein–specific antibodies/nos. positive in H5-specific hemagglutination inhibition assay (titer ≥16).
§Combined data on proportion of deaths for barns A/10 and A/13; increased losses in barn A/10 occurred after early August 2007.
#Date of regular slaughter. HPAIV (H5N1) was found in retained frozen duck carcasses of flocks B/3–5 (hatched 6/20/07) and subtype H5N1–specific RNA was present in 1 frozen duck carcass of flock B/1–1 (hatched 6/29/07), but the sample could not be pathotyped due to low viral genome loads.
¶Due to low viral loads the H5N1 pathotype could be confirmed by sequencing a hemaglutinin fragment in only 2 cases.

an initial virologic investigation of 60 oropharyngeal and cloacal swabs yielded negative results. However, residual lung tissues obtained from 2 retained frozen carcasses of ducks that had been reared at farm B (barn B/3–5) and slaughtered at the abattoir at farm A on August 1, 2008, tested positive for HPAIV (H5N1) (Table). In this fattening flock, a slightly increased cumulative proportion of deaths (8.3%) was evident (Figure 1, panel B). After these findings, swab sampling was increased to 450 per barn unit at farms B and C to ensure detection of HPAIV infection at a prevalence of 1% with 99% confidence. In addition, serologic surveillance was initiated. No evidence for any infection by AIV H5 was found at farm C (Table). Farm B, however, housed at least 1 flock of ducks ready for slaughter and marketing (Table: B/2–4) that showed serologic evidence for widespread infection with AIV H5. In 4 oropharyngeal swabs of this flock, low genome loads of AIV (H5N1) were detected; 2 swabs yielded sufficient material to confirm, by sequencing, the presence of HPAIV. The low prevalence of active viral infection contrasted the high H5-specific seroprevalence, which indicated that the peak of infection in this flock had passed probably 2–3 weeks before swabbing for virologic testing had been initiated on September 5, 2007 (Table). The overall cumulative losses in this flock nevertheless amounted to only 5.9% (Table; Figure 1, panel D). The culling of poultry on farms B and C was completed on September 10, 2007, and all poultry

of farms B and C slaughtered after July 31, 2007, at the abattoir at farm A were confiscated and destroyed.

Retrospective analysis of duck deaths at farm B showed modestly enhanced cumulative values in at least 3 fattening flocks during midterm or toward the end of the fattening period (Table; Figure 1, panels A–C). Virologically, HPAIV (H5N1)–positive animals were detected retrospectively in barn B/3–5, which had hatched on June 20, 2007, and showed a cumulative proportion of deaths of 8.3% (Table; Figure 1, panel B). Inconclusive results were obtained for the flock that had hatched on June 29, 2007 (Table; Figure 1, panel C). Another suspected fattening flock (hatching date April 25, 2007; Table; Figure 1, panel A) could not be retrospectively analyzed. In none of these flocks did daily mortality rates exceed 2%, the legal cut-off for mandatory targeted etiologic investigations including for avian influenza viruses. However, cumulative proportion of deaths amounted to up to 20.3%.

Brandenburg

On December 10, 2007, three of 11 chickens were found dead at a backyard holding in the Federal State of Brandenburg in northeastern Germany. Another chicken had died on December 7, and a reduction in egg production on the farm was observed. HPAIV (H5N1) was detected in 2 birds submitted for pathologic and laboratory testing on December 11. Clinical signs in the chickens that were
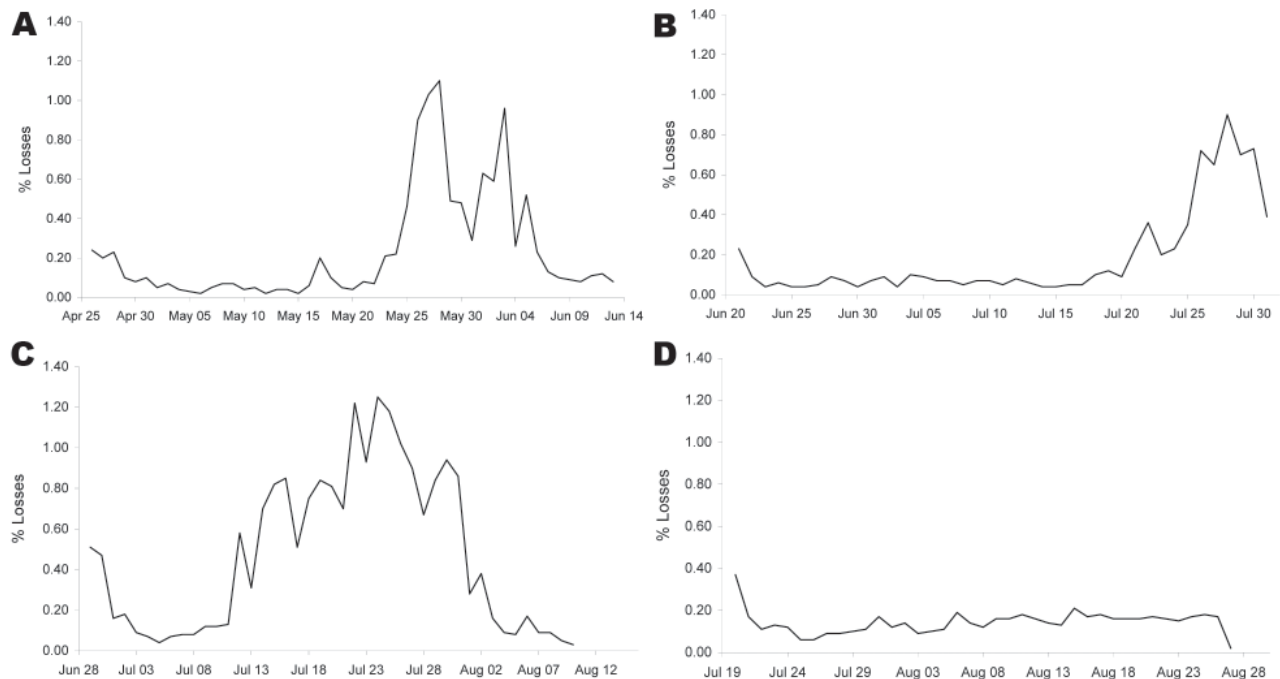
Figure 1. Deaths of ducks in different barn units of farm B, Bavaria. A) Barn B/3–5; duck hatched April 25, 2007, slaughtered June 14, 2007, at an abattoir in Lower Saxony: no material available for testing. B) Barn B/3–5; duck hatched June 20, 2007, slaughtered August 1, 2007, at farm A): 2 highly pathogenic avian influenza virus (HPAIV) A (H5N1)–positive samples detected in retained samples of frozen duck carcasses; virologic and serologic results suggestive of a recent HPAIV (H5N1) infection are listed in the Table. C) Barn B/1–1; duck hatched June 29, 2007, slaughtered August 11, 2007, at farm A; subtype H5N1–specific RNA was detected in 1 retained sample of a frozen duck carcass, but the pathotype could not be determined due to low viral loads. D) Barn B/2–4; duck hatched July 20, 2007, culled September 9, 2007; virologic and serologic results suggestive of a recent HPAIV (H5N1) infection are listed in the Table.

still alive on December 12 and 13 included lethargy, ruffled feathers, reduced mobility, and cyanosis of the combs and wattles. Two of these birds died on December 12, and another 3 died on December 13. The remaining chicken was culled on December 14 after HPAIV (H5N1) had been confirmed in samples submitted to the national reference laboratory on that day. Two more cases of HPAIV (H5N1) infection in similar backyard chicken holdings were detected on December 20 and 23, 2007, respectively, in the same region but separated by 80–120 km.

No direct connections between these holdings were identified. As judged from the lack of recent movements of animals, vehicle traffic, and contacts of owners, no hints toward an incursion or further spread of virus was evident through these routes. Holdings were situated in areas rich in migratory birds. In addition, only 1 further condition appeared to be shared between these holdings: Within 2 to 4 days before the outbreaks, the chickens had access to raw offal of deep-frozen duck carcasses that had been purchased from a supermarket chain in October in that region. These ducks had been frozen and were consumed just 3–5 days before the outbreaks. Owners of the third holding refused to make any specific comments concerning this point, but

circumstantial evidence points toward a similar scenario. At the time of investigation, no further material from any of these deep-frozen duck carcasses was available for analysis.

Outbreak-associated Surveillance Activities

Bavaria

From January 1, 2007, until August 31, 2007, a total of 1,236 wild birds were tested in Bavaria for avian influenza viruses. From June 24 through August 3, 2007, HPAIV (H5N1) was detected in 19 aquatic birds (mute swans, gray lag and Canada geese, tufted ducks). Extensive serologic (2,107 samples) and virologic (5,833 samples) surveillance in altogether 46 further contact holdings to farm A extending over the whole area of Germany did not yield any indication of past or ongoing AIV H5 infections.

Brandenburg

In 2007, a total of 1,696 wild birds were tested in Brandenburg for avian influenza virus. In December 2007 and in January 2008, 283 and 162 wild birds, respectively, were tested with negative results. HPAIV (H5N1) was not

detected in any of the samples. Serologic testing of 4,040 blood samples and virologic testing of 2,836 swab samples from poultry had negative results for subtype H5N1 in 2007.

Phylogenetic Analyses

The HA gene of 1 representative virus isolate of each of the 3 Brandenburg holdings and of the virus isolated from duck meat were sequenced. Sequence comparisons showed a very close relationship between viruses from the 3 Brandenburg holdings and from the Bavarian duck meat. Within the HA gene, complete identity was found between 2 viruses in the Brandenburg holdings and the virus in duck meat; the third Brandenburg virus was distinguished by a single nonsynonymous mutation (K65R).

Phylogenetic analysis of the full-length HA gene of these and other HPAIV (H5N1) viruses isolated in 2007 in Germany and neighboring countries is presented in Figure 2. All viruses belonged to cluster 2.2, group C. This lineage had not been detected during the 2006 outbreaks among wild birds in Germany and, therefore, most likely represents a new incursion in 2007 (*7*). Viruses from the Bavarian holding A clustered separately from those of the Bavarian holding B. Brandenburg viruses A–C clustered with the virus sequences from duck meat originating from Bavarian holding B (Figure 2).

Discussion

The high homology of RNA sequences of the HA genes derived from viruses that caused outbreaks at 3 different holdings in the German Federal State of Brandenburg and virus isolated from deep-frozen duck carcasses (A/duck meat/Bavaria/2048/2007) clearly points to a close epidemiologic link between these outbreaks. According to theoretical consideration regarding the mutation rate of influenza A viruses (*14*) and to practical experiences during

outbreaks (*4*), if HPAIV had gone through a transmission chain consisting of several hosts, more extensive sequence differences would likely have resulted. This likelihood is further emphasized by the comparison of 3 subtype H5N1 virus isolates derived from a single barn of farm A (Figure 2); these viruses exhibit greater variability among each other than do viruses from the 3 different affected backyard holdings in Brandenburg. Also, distinct sequences of viruses were detected from poultry in Poland, where outbreaks occurred at the same time as the Brandenburg cases, which renders an incursion from this source highly unlikely. Among the Brandenburg cases, no epidemiologic links could be detected except that, as proven in 2 cases and assumed to have occurred in the third case, backyard chickens had access to uncooked offal from duck meat purchased separately in different supermarkets of the same national chain.

Oral uptake of virus is an efficient way of transmitting HPAIV among poultry and mammals (*15,16*). Infectious virus in titers of up to $10^{7.2}$ 50% egg infectious doses per gram in muscles of infected chickens, ducks, and quails has been repeatedly demonstrated (*17,18*). Although feeding of poultry offal to poultry or livestock is legally prohibited in Germany, unintended access of backyard poultry to poultry meat and organs is sometimes possible. If such offal is contaminated with HPAIV, transmission becomes possible, and isolated outbreaks like those reported from Brandenburg may ensue. However, this circumstance would require the presence of HPAIV in meat destined for human consumption. This possibility had previously been estimated to be low (*19*).

Unfortunately, no experimental evidence could be produced that unequivocally links the Bavarian farm B and Brandenburg backyard outbreaks because no material was left for virologic examination from the suspected deep-frozen duck carcasses. Therefore, we tried to collect

Figure 2. Phylogenetic tree of the hemagglutinin (HA) gene (full-length sequence) of highly pathogenic avian influenza virus (HPAIV) (H5N1) detected in poultry from Brandenburg and Bavaria, Germany, in 2007, including sequences of wild birds and poultry from neighboring countries. Sequence of the Czech poultry isolate is supported by GenBank. The tree was constructed by using a minimal-evolution algorithm; numbers represent bootstrap values after 1,000 replications. A maximum-likelihood (ML)–based tree resulted in a similar topology; italicized numbers indicate bootstrap values of the ML tree after 200 replications. Scale bar indicates substitutions per site. The HA sequence of A/chicken/Czech Republic/11242–38/2007 (H5N1) was extracted from GenBank accession no. EU 443553. LPAIV, low pathogenicity avian influenza virus.

further epidemiologic data by fully retracing the origin of the frozen ducks purchased by the owners of the backyard chickens in Brandenburg. This attempt included authorities in the involved Federal States and the management of the supermarket chain that had sold the duck meat.

The duck meat sold in autumn 2007 by the supermarket chain in Brandenburg and in the neighboring Federal States of Berlin and Mecklenburg–Western Pomerania had been purchased from a slaughterhouse in the Federal State of Lower Saxony (lots 724, 725; Figure 3). Direct links between Bavarian farm B and the slaughterhouse in Lower Saxony existed because slaughtering lots 724 and 725 included ducks from 3 fattening flocks from farm B. These ducks were slaughtered on June 14, 19, and 22, 2007, in the abattoir in Lower Saxony, because the most frequently used abattoir in Bavaria at farm A was closed for holidays (Figure 3). Among the 3 flocks was the one from barn B/3–5, which showed a suspiciously elevated cumulative proportion of deaths of 12.6% (Table; Figure 1, panel A).

This circumstantial evidence points toward a transient and limited incursion of HPAIV (H5N1) into duck meat destined for human consumption. Therefore, enhanced virologic screening of fattening ducks has been initiated in Germany. Also, reporting obligations related to deaths in duck flocks were adopted. Since the end of the Brandenburg outbreaks, a single, unrelated reccurrence of HPAIV (H5N1) in Germany was detected in poultry in 2008, but none has been detected in wild birds. To date, no clinical cases of human infection with subtype H5N1 have been reported in Germany.

In avian hosts, the clinical picture of an HPAIV infection depends, among other factors, on the species affected (20). In particular, domestic waterfowl showed substantial variations in clinical features resulting from infection with strains of HPAIV (H5N1) of Asian origin (21). Factors influencing the clinical course relate to species, age of animals, and the virus strain (22,23). The cited experimental data as well as reported evidence from the field (24) show that an introduction and subsequent spread of HPAIV (H5N1) in duck flocks is likely clinically silent. Also in the cases reported here, hardly any clinical symptoms suggestive of an HPAIV infection, in particular, neurologic manifestations, were evident. Increased proportion of deaths, as seen in some of the described duck-fattening flocks, might have been fueled by bacterial co-infections, e.g., by those caused by *Riemerella* spp. Through silently but productively infected ducks, an endemic status of HPAIV (H5N1) infection can be established and perpetuated (25). Strains isolated from such endemic infections induce no clinical symptoms in ducks but retain high pathogenicity for chickens and turkeys. No sign of even widespread infection would be clinically apparent until the virus has become more established in flocks of highly
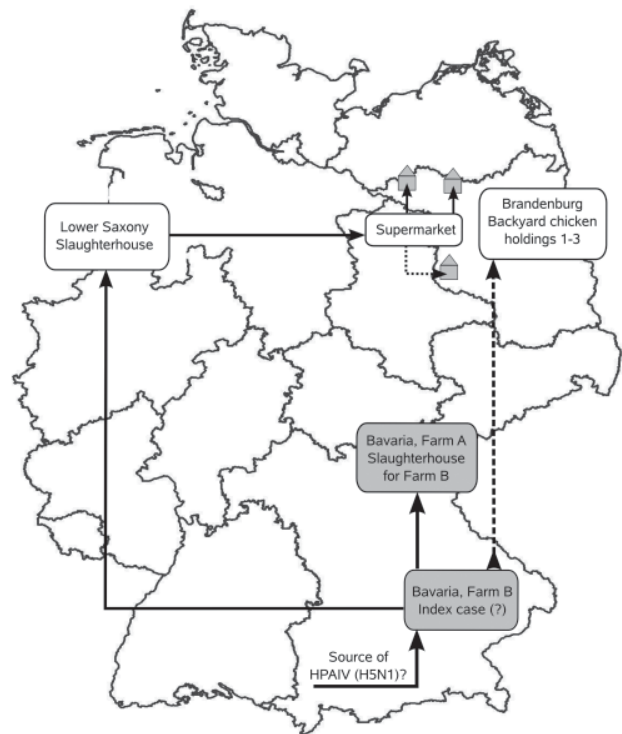

Figure 3. Possible pathway of transmission of highly pathogenic avian influenza virus (HPAIV) (H5N1) from farm B, Bavaria, to 3 backyard chicken holdings in Brandenburg (gray house symbols) based on phylogenetic and circumstantial epidemiologic evidence. Viruses of these cases were virtually identical, although they were separated by 4 months (August and December, 2007) and ≈400 km without linking outbreaks. In contrast, other viruses occurring at the same time (August) in Bavaria in wild birds or in farm A were distinguishable from those of farm B. The same was true for viruses detected in Poland (close to Brandenburg) in December. Therefore, a direct epidemiologic link between farm B and the outbreaks in Brandenburg was suspected (dashed arrow). From June 14 through June 22, 2007, three fattening flocks from farm B were slaughtered in Lower Saxony (angled arrow). These included flock B/3-5 with elevated proportion of deaths (Figure 1, panel A). Slaughtering lots 724/725, which contained ducks from farm B flocks with an elevated cumulative proportion of deaths, were distributed in Brandenburg supermarkets (horizontal arrow). Frozen duck carcasses from these lots had been purchased by the owners of the Brandenburg chicken holdings, and in 2 of the 3 outbreaks, owners admitted that chickens had access to uncooked offal from these carcasses before the outbreaks.

vulnerable (gallinaceous) species (26). Such mechanisms are obviously not restricted to subtropical Southeast Asia.

In conclusion, our data show that incursions of recent strains of descendants of Qinghai lineage HPAIV (H5N1) strains into industrial duck-fattening holdings in Europe may be clinically silent, even in young ducklings. If daily mortality rates remain low, an increase in cumulative mortality may still be evident and should prompt specific virologic investigations. Undetected HPAIV (H5N1)

infections of domestic waterfowl destined for human consumption raises the risk for human infection when infected birds or contaminated meat products are handled. Thus, measures must be strengthened that can prevent this zooanthroponotic virus from entering the food chain through contaminated duck meat products and spreading further. Intensified monitoring of duck herds for HPAIV infection that does not rely on syndromic surveillance would be required first. In the outbreaks reported here, the power of serologic assays to detect virus incursions is notable, compared with results by rRT-PCR. Species-independent cEIA assays detecting antibodies specific for the nucleocapsid protein of influenza A viruses were more sensitive than HI assays that used 2 different H5 antigens. Despite the intrinsically higher sensitivity of most ELISAs, the discrepancy in this study might have been aggravated by not using antigen of the outbreak viruses in HI assays. Nevertheless, cEIAs would be suitable for high-throughput analysis in extensive monitoring programs while HI techniques would not.

Acknowledgments

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Zoonotic Infections

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European Bat Lyssavirus Transmission among Cats, Europe

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We identified 2 cases of European bat lyssavirus subtype 1 transmission to domestic carnivores (cats) in France. Bat-to-cat transmission is suspected. Low amounts of virus antigen in cat brain made diagnosis difficult.

Most countries in western Europe are currently free of rabies in terrestrial mammals, as was the case in France during 2001–2008 (1). However, rabies still remains a public health problem in these countries because of natural circulation of bat-specific viruses (order Mononegavirales, family *Rhabdoviridae*, genus *Lyssavirus*) such as European bat lyssaviruses (EBLVs). These viruses are divided into genotypes 5 (EBLV-1) and 6 (EBLV-2); the first genotype is subdivided into subtypes a and b (2). Knowledge of the prevalence and epidemiology of EBLV is limited (2–5). To date, natural transmission of EBLV-1 has been reported in a limited number of terrestrial mammals, including 5 sheep in Denmark (6) and 1 stone marten in Germany (7) (Table 1). Since 1985, only 3 human deaths from EBLVs have been confirmed (3) (Table 1). We describe 2 documented cases of spillover transmission of EBLV in domestic carnivores (cats, *Felis domesticus*) in Europe.

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The Study

In November 2003, a 6-month-old female stray cat (cat no. 1) was found ill in a public garden in Vannes (Morbihan District) in western France and taken to a veterinary clinic. This animal had convulsions and moderate dehydration and was emaciated. It was infected by feline immunodeficiency virus, which was compatible with the clinical symptoms. The veterinarian was bitten while providing veterinary care to the cat. After a few days, the cat recovered and was impounded for veterinary surveillance. It died suddenly the following night. No information about potential contact with bats was available.

On November 8, 2007, an 18-month-old female cat (cat no. 2) was taken by its owner to a veterinarian in Fontenay-le-Comte (Vendée District) in western France because of abnormal behavior. The owner reported having been bitten by the cat. The next day, the cat showed severe central neurologic disorders and aggressive behavior. It died during the next night. Its outdoor access appeared to have been restricted. Two months later, the carcass of a bat (*Eptesicus serotinus*) was recovered in the same area of Fontenay-le-Comte and submitted for rabies testing.

Recommended techniques for rabies diagnosis were used for all animals (8). For cat no. 1, results of a repeated direct immunofluorescence antibody test (FAT) with a polyclonal antirabies conjugate (Bio-Rad, Marnes-la-Coquette, France) performed on different cortex and spinal bulb smears were negative. Viral isolation by using a rabies tissue culture infection test (RTCIT) was also unsuccessful, as was attempted isolation of virus by using a mouse inoculation test (MIT) (Table 1). The only test routinely used that gave a positive result was an antigen-capture ELISA (WELYSSA) for lyssavirus antigen (9). The presence of EBLV RNA (03011FRA) was determined by reverse transcription–PCR (RT-PCR) targeting short viral gene regions (5).

Lyssavirus antigens were repeatedly detected by FAT in different areas of the brain of cat no. 2. Viral isolation by using RTCIT was positive only after the second cell culture passage. Results for isolation of EBLV (07240FRA) by MIT were positive. Lyssavirus antigen detection by WELYSSA was variable, depending on the part of the brain tested. Viral RNA was detected by RT-PCR (Table 1). The bat was positive for EBLV by FAT, RTCIT (08120FRA), MIT, and RT-PCR.

Nucleotide sequencing and phylogenetic analysis identified isolate 03011FRA as EBLV-1b and isolates 08120FRA and 07240FRA as EBLV-1a (Figure 1). Sequencing of the complete genome (10) of the 2 EBLV-1a isolates showed a high percentage of homology (Table 2).

After identification of these 2 cases of spillover transmission of EBLV-1 to domestic cats, postexposure prophylaxis measures were implemented. The veterinarian who

Table 1. Confirmed cases of EBLV spillover transmission to terrestrial mammals and humans, Europe*

Host (no. cases)	Year of isolation	Location	Clinical signs or disease	Techniques used for rabies diagnosis on original brain samples					EBLV type
				FAT	RTCIT	MIT	ELISA	RT-PCR	
Sheep† (4)	1998	Western Jutland, Denmark	Neurologic disorders	+ (weak)	+ (1 of 4 sheep)	–	ND	+ (only 1 sheep tested)	1a
Stone marten‡ (1)	2001	Burg, Saxony-Anhalt, Germany	No obvious clinical signs	– (repeated testing)	+ (weak)	+	ND	+	1a
Sheep† (1)	2002	Western Jutland, Denmark	Neurologic disorders	+	+	–	ND	ND	1a
Domestic cat§ (1), cat no. 1	2003	Vannes, Morbihan, France	Emaciated, moderate dehydration, FIV detected	– (repeated testing)	– (after 3 cell culture passages)	–	+	+	1b
Domestic cat§ (1), cat no. 2	2007	Fontenay-le-Comte, Vendée, France	Neurologic disorders, aggressive	+ (weak)	+ (weak after 2 cell culture passages)	+	+ (variable)	+	1a
Man, 30 y of age (1)	1985	Helsinki, Finland	Rabies	+	+	+	ND	ND	2
Girl, 11 y of age (1)	1985	Belgorod, Russia	Rabies	–	ND	+	ND	ND	1a
Man, 55 y of age (1)	2002	Angus, Scotland	Rabies	+	+	+	ND	+	2

*EBLV, European bat lyssavirus; FAT, direct immunofluorescence antibody test; RTCIT, rabies tissue culture infection test; MIT, mouse inoculation test; RT-PCR, reverse transcription–PCR; ND, not determined; FIV, feline immunodeficiency virus.
†*Ovis aries*.
‡*Martes foina*.
§*Felis domesticus*.

was bitten by cat no. 1 received a booster rabies vaccination, and 15 persons exposed to cat no. 2 during the 2-week critical period before its death received appropriate postexposure treatment on the basis of national and international recommendations of the World Health Organization (*8*,*11*). Two family dogs potentially exposed to cat no. 2 and previously vaccinated received a booster vaccination. Cross-neutralization data obtained with human serum samples and in rodent models suggest that preexposure and postexposure treatments for rabies are effective against EBLV-1.

Control measures were implemented to prevent potential further contaminations, although cats represent naturally dead-end host for rabies (and for lyssavirus), thereby limiting any risk for transmission to other mammals. City authorities conducted a census of all domestic animals in the neighborhood where cat no. 2 lived. All cats, dogs, and ferrets were identified by microchips and kept under veterinary surveillance. Dogs had to be leashed and cats kept indoors during the next 2 months.

Conclusions

We report 2 documented cases of natural infection of domestic cats by EBLV-1 lyssaviruses presently circulating in European bats (*2–5*). Our study demonstrates that subtypes EBLV-1a and EBLV-1b can cross the species barrier, although cat no. 1 probably died of feline leukemia. However, cat no. 2 died with neurologic signs compatible with rabies and was positive for EBLV by FAT, the reference technique. Direct transmission of EBLV-1 from bats

to cats seems the most realistic explanation for these cases because cats prey on bats and have numerous contacts with them (*3*,*12*,*13*). Identification of a highly homologous EBLV-1a isolate from a rabid bat found in the same location as cat no. 2 supports this hypothesis.

Difficulties in EBLV-1 detection in the brain of these 2 cases of spillover transmission are reminiscent of transmission previously reported (*6*,*7*) (Table 1), potentially caused by a low amount of virus antigen in the brain. This finding further underlines the importance of using different techniques to diagnose rabies caused by EBLVs (*9*). This issue raises questions about the true incidence of these viruses among cats.

At the Institut Pasteur during 1997–2007, a total of 6,097 cats suspected of having rabies and originating from all districts in France showed negative results for rabies by 2 recommended techniques (FAT and RTCIT). Among them, all animals tested since 2004 (1,506 cats), except cats no. 1 and no. 2, were also negative for rabies by WELYS-SA (Figure 2), which suggests that transmission of EBLVs from bats to cats, although possible, is rare. Furthermore, terrestrial mammals seem to represent dead-end hosts for EBLVs, as suggested by results of experimental EBLV inoculations in several mammals such as cats, dogs, ferrets, mice, red foxes, or sheep (*6*,*7*,*14*). These animals are susceptible to infection with EBLVs but seem unlikely to actively transmit EBLVs to a new host.

Comparative analysis of the full-length genomic sequence of the EBLV-1a from cat no. 2 isolate 07240FRA
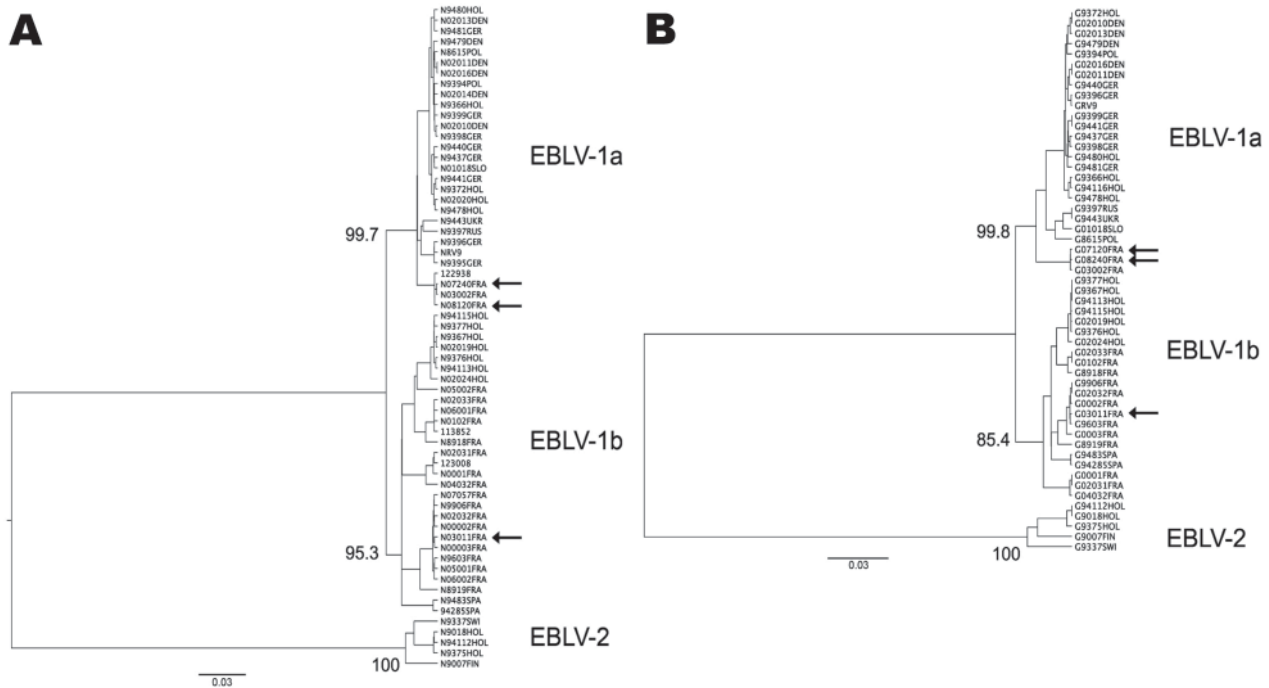
Figure 1. Phylogenetic tree comparing nucleotide sequences of A) nucleoprotein (372 nt, position 63 from the translation initiation site) and B) glycoprotein (547 nt, position 640 from the translation initiation site) genes of spillover transmission of European bat lyssavirus-1 (EBLV-1) in terrestrial mammals and human with representative isolates of the diversity of EBLV-1 in Europe. Cases described in this report are indicated by the arrows. For each dataset, we inferred a maximum clade credibility phylogenetic tree by using the Bayesian Markov Chain Monte Carlo method available in the Bayesian Evolutionary Analysis Sampling Trees software (http://beast.bio.ed.ac.uk). This analysis used a relaxed (uncorrelated lognormal) molecular clock and the HKY85 + Γ_4 model of nucleotide substitution. All horizontal branches are scaled according to the number of substitutions per site. Bootstrap values are indicated at the nodes. All GenBank accession numbers corresponding to full-length or partial nucleoprotein and glycoprotein nucleotide sequences were previously described (2,10) except for nucleoprotein nucleotide sequences of isolates 03011FRA (EU636795), 04032FRA (EU636794), 05001FRA (EU636790), 05002FRA (EU636789), 06001FRA (EU636791), 06002FRA (EU636792), and 070057FRA (EU636793) and glycoprotein nucleotide sequences of isolates 03011FRA (EU636787) and 04032FRA (EU636788).

Table 2. Percentage nucleotide divergence between EBLV-1a strains isolated from a cat (07240FRA) and bats (08120FRA, 03002FRA, and RV9)*

Gene	Sequence	Strain		
		07240FRA/08120FRA	07240FRA/03002FRA	07240FRA/RV9
Complete genome	nt	0.1 (12)	0.4 (36)	2 (226)
Nucleoprotein	nt	0	0.2 (2)	1.5 (19)
	aa	0	0	0
Phosphoprotein	nt	0.1 (1)	0.4 (4)	2.3 (23)
	aa	0.3 (1), **I149T**	0.7 (2), **I149T**, **G175D**	1.7 (7), **Q147R**, **I149T**, **T156A**, **F169S**, **P174L**, **G175D**, **G266S**
Matrix	nt	0	0.3 (2)	1.5 (7)
	aa	0	0	1 (2), **N2K**, **I155M**
Glycoprotein	nt	0	0.2 (3)	2.1 (32)
	aa	0	0.2 (1), **S489P**	0.8 (4), **L244Q**, **S278N**, **S489P**, **A521T**
Polymerase	nt	0.03 (2)	0.3 (16)	1.9 (109)
	aa	0	0.2 (4), **A97T**, **G1160D**, **T1754I**, **R1894S**	0.3 (5), **R315K**, **I391V**, **K980R**, **T1754I**, **R1894S**

*Strains 07240FRA and 08120FRA were isolated from cat no. 2 and from a bat (*Eptesicus serotinus*) found dead in the same area (Fontenay-le-Comte, France), respectively. Strain 03002FRA was isolated from another bat (*E. serotinus*) collected ≈100 km from Fontenay-le-Comte in 2003 (10). Isolate RV9 was collected from a bat (*E. serotinus*) in 1968 in Germany (15). Numbers of substitutions are indicated in parentheses. Type of amino acid substitutions are indicated in **boldface**. GenBank accession numbers for full-length genomic sequences corresponding to strains 07240FRA, 08120FRA, 03002FRA, and RV9 are EU626552, EU626551, EU293109, and EF157976, respectively. EBLV, European bat lyssavirus; nt, nucleotide; aa, amino acid.
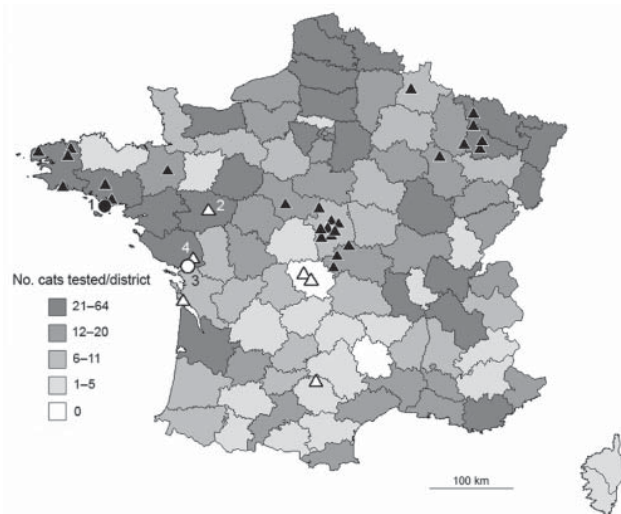
Figure 2. Distribution of cats analyzed during 2004–2007 and of bats found positive for European bat lyssavirus (EBLV) in France during 1989–2007. Distribution of 1,506 cats tested during 2004–2007 by direct immunofluorescence antibody test, rabies tissue culture infection test, and an antigen-capture ELISA is given by district. Precise location of the 2 infected index (positive) cats and positive bats (n = 32) are indicated by circles and triangles, respectively, and associated with numbers 1, 2, 3, and 4 for isolates 03011FRA, 03002FRA, 07240FRA, and 08120FRA, respectively. EBLV-1a and EBLV-1b isolates are indicated in black and white, respectively. Map was constructed by using Articque's C&D software (www.articque.com) and published according to Articque's publication policy.

with bat isolate 08120FRA and with another bat (*E. serotinus*) isolate (03002FRA) collected in 2003 ≈100 km from Fontenay-le-Comte showed high similarity (Table 2). This finding indicates that heterologous passage of EBLV-1a in a cat did not select mutants. Similarly, the lower similarity observed with a genomic sequence collected from a bat RV9 (*15*) (*E. serotinus*) in 1968 in Germany also indicates that EBLV-1 evolution is shaped by slow genetic drift (*2*).

No secondary cases originating from cat no. 1 and cat no. 2 were reported (after 6 months of follow-up for cat no. 2). However, improving surveillance and raising awareness to better understand the epidemiology of lyssaviruses are necessary. Persons bitten by bats or by any carnivorous animal are advised to wash wounds with water and soap and to seek medical attention (*8,11*).

Acknowledgments

Dr Dacheux works in the UPRE Lyssavirus Dynamics and Host Adaptation unit at the Institut Pasteur in Paris and at the National Reference Centre for Rabies in Paris. His research interest is rabies, particularly its surveillance, development of new diagnostic tools, and physiopathologic studies in humans and animals.

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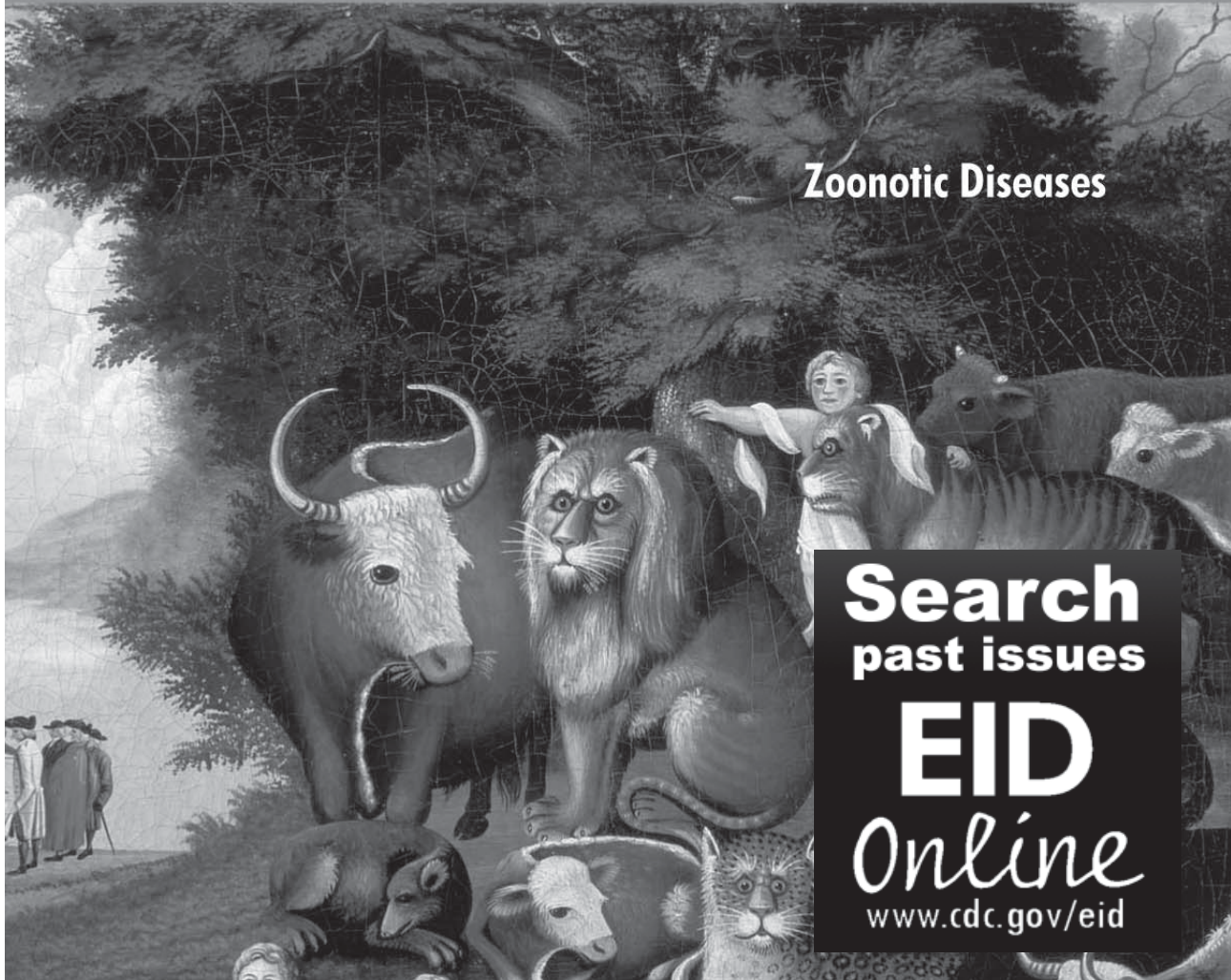
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Zoonotic Diseases

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Staphylococcus aureus ST398, New York City and Dominican Republic

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Patricia Leon, and Franklin D. Lowy**

Closely related *Staphylococcus aureus* strains of ST398, an animal-associated strain, were identified in samples collected from humans in northern Manhattan, New York, NY, USA, and in the Dominican Republic. A large population in northern Manhattan has close ties to the Dominican Republic, suggesting international transmission.

In the past 5 years, as methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a community pathogen, awareness of the role of animal exposure from pets or farming as sources of MRSA has increased (*1–3*). We identified a clone of *S. aureus* previously associated with outbreaks of infections in animals and in humans who work with animals in 2 unique collections of *S. aureus* isolates. The first was from a population-based study of *S. aureus* colonization among residents of northern Manhattan in New York, NY, USA; the second was from isolates obtained from the Dominican Republic. This clone does not digest with the restriction enzyme *Sma*I, which is generally used for pulsed-field gel electrophoresis (PFGE). Consequently, the clone is identified by multilocus sequence typing as sequence type 398 (ST398). Both methicillin-resistant and methicillin-susceptible isolates of *S. aureus* have been reported (*4*). ST398 has been found primarily in Europe, where it has been isolated from pigs and pig farmers in the

Netherlands and France and from dogs, pigs, horses, and humans in Germany and Austria (*5–8*). Colonization with MRSA ST398 has recently been reported in pigs and pig farmers in Canada (*9*).

The Study

The community-based study was conducted from 2004 through 2007 in the northern section of Manhattan, a borough of New York City. Northern Manhattan contains a large, medically underserved population that has close ties to the Dominican Republic. Participants were recruited by using random-digit dialing. Consenting persons and household members were subsequently interviewed and screened for *S. aureus* colonization. A total of 321 eligible households containing 914 household members participated. In 9 households, 13 participants were found to be colonized with *S. aureus* isolates that were *Sma*I resistant. Digestion with the *Cfr*9I, an isoschizomer of *Sma*I, yielded identical PFGE profiles (Figure). Subsequent multilocus sequence typing confirmed the ST398 identification (allelic profile 3–35–19–2-20–26–39). All strains were methicillin susceptible. A representative strain was *spa*-typed as type t571 (allelic profile 8–16–2-25–2-25–34–25, eGenomics type 109); it was Panton-Valentine leukocidin negative.

Characteristics of persons colonized with ST398 were similar to those of persons in the community-based study and with northern Manhattan census characteristics (Table). The 13 isolates were from 9 different families; 1 family had 4 members colonized with ST398 at either nasal or axillary sites. The mean age of those colonized was 33.4 years; only 1 child (7 years of age) was colonized. Two persons from different families were colonized with ST398 at multiple sites, none of which were confirmed as infections.

No household reported owning pets, although 2 reported animal contact. Of the 12 adults, 5 (41.7%) reported possible job exposure to *S. aureus*, including 1 who worked in a healthcare-associated field. No household reported patronizing *viveros*, or live poultry markets, which are common in the Latino communities of northern Manhattan and the Bronx. Two households reported having children who attended day care, although none of these children were colonized with *S. aureus*. Although 15% of the Dominican population in the study reported travel to the Dominican Republic within 6 months of their interview, none of the colonized participants reported recent travel to the Dominican Republic. No contact among the different households was reported.

A second collection of *S. aureus* isolates was gathered during 2007 and 2008 from a convenience sample of 89 anonymous infection and colonization isolates received from the Dominican Republic. Six isolates were identified as methicillin-susceptible *S. aureus* clone ST398. Strains were provided by 1 hospital in Santo Domingo, Dominican
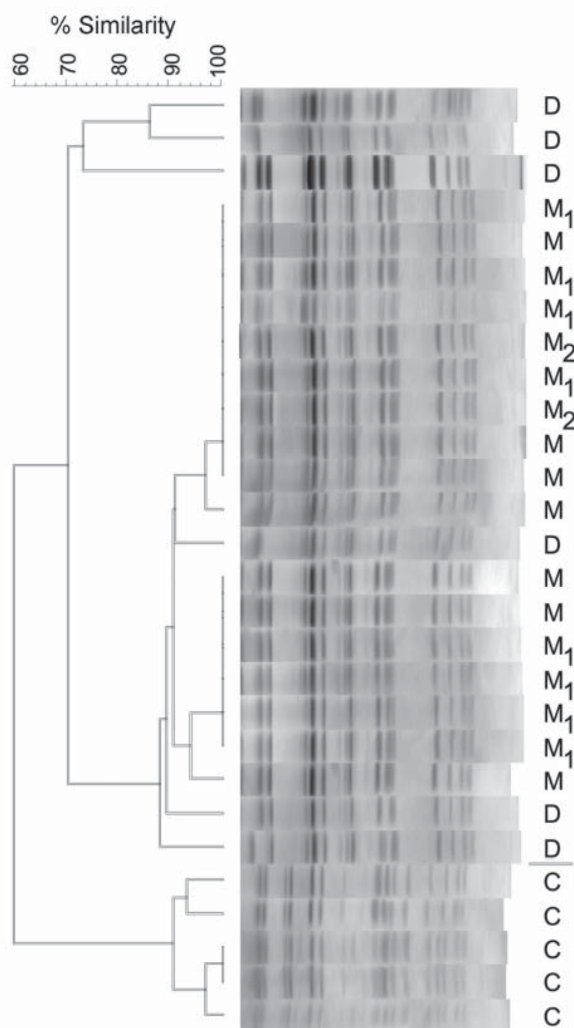
Figure. Pulsed-field gel electrophoresis profiles of sequence type 398 isolates from the Dominican Republic (D); northern Manhattan (M), New York, NY, USA; and Canada (C) (provided by Scott Weese). Strains within households in which >1 person was colonized are identified numerically. The dendrogram shows the percent similarity of the isolates. A similarity ≥70% indicates closely related or identical strains.

Republic (n = 53), and 1 microbiology laboratory, the Laboratorio Referencia (n = 28), which serves as a reference laboratory for the country. Four isolates from the hospital and 2 from Laboratoria Referencia were identified as methicillin-susceptible ST398. Sociodemographic data on these persons were limited. Two of the ST398 isolates were from women with infections living in Santo Domingo, and the remaining 4 were colonization samples. Of the 6 isolates, 5 were found to be *spa*-type t571 (eGenomics type 109), and 1 was found to be type t3625 (eGenomics untyped).

Pairwise similarity scores for the isolates were calculated by the Dice coefficient, and an overall similarity score was calculated by using the unweighted pair group method with arithmetic mean. Comparing the isolates by using a dendrogram-based similarity score ≥70%, we found that the strains from northern Manhattan and from the Dominican Republic were closely related, although they contrasted with ST398 isolates from Canada (provided by Scott Weese, Ontario Veterinary College, University of Guelph) (Figure) (*10*).

Conclusions

Identification of the *S. aureus* clone ST398 in northern Manhattan and in the Dominican Republic suggests its introduction into the United States by travelers between the 2 countries. The largely Latino population of northern Manhattan is composed mainly of immigrants or first-generation families from the Dominican Republic; travel between the 2 regions is common. Alternatively, northern Manhattan may contain reservoirs, such as live poultry markets, which may serve as a means of strain transmission.

Colonization or infection with the *S. aureus* clone ST398 has been associated with exposure to pigs, pets, and other animals (*5,7–9*), and the *S. aureus* clone ST398 has been isolated from meat products (*2*). However, transmission is not limited to animal exposures. Person-to-person spread has occurred among household members and in the hospital setting (*6,8,11*). For example, a dramatic increase in persons colonized as well as infected with MRSA clone ST398 was recently reported in a Dutch hospital (*12*).

The presence of this strain among several household members in our study reinforces earlier observations of the potential for horizontal transmission of this clone after it is introduced into an appropriate setting. Although information about the persons from the Dominican Republic was limited, the 2 groups provided identical strain profiles, suggesting a possible link between the 2 countries. Given ST398's history of rapid dissemination in the Netherlands, its potential for the acquisition of methicillin resistance, and its ability to cause infections in both community and hospital settings, monitoring the prevalence of this strain in northern Manhattan and the Dominican Republic will be important to understand more about its virulence and its ability to spread in these communities.

Table. Characteristics of persons colonized with *Staphylococcus aureus* ST398, northern Manhattan, New York City, NY, USA, 2004–2007, compared with study population and 2000 census population for area*

Characteristics	ST398 subset, no. (%)	Overall study population, no. (%)	2000 census population, no. (%)
Race/ethnicity			
Latino	11 (84.6)	813 (89)	173,755 (68)
Non-Hispanic white	2 (15.4)	90 (9.8)	65,449 (25.6)
African American	0	11 (1.2)	53,514 (20.9)
Asian	0	0	5,370 (2.1)
Sex			
Male	4 (30.8)	362 (39.6)	120,866 (47.3)
Female	9 (69.2)	552 (60.4)	134,723 (52.7)
Age group, y			
<5	0	84 (9.2)	17,878 (7.0)
5–17	1 (7.6)	238 (26.2)	49,196 (19.3)
18–44	10 (76.9)	297 (32.5)	112,195 (44.0)
≥45	2 (15.4%)	290 (31.9)	76,320 (29.9)
Occupational exposure	5 (38.5)	58 (6.4)	NA
Travel outside USA	7 (53.8)	171 (18.7)	NA
Daycare exposure	2 (15.4)	87 (9.5)	NA
Total population	13 (100)	914 (100)	255,589 (100)

*ST, sequence type; NA, not available.

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Genomic Characterization of Novel Human Parechovirus Type

Linlin Li, Joseph Victoria, Amit Kapoor, Asif Naeem, Shahzad Shaukat, Salmaan Sharif, Muhammad Masroor Alam, Mehar Angez, Sohail Zahoor Zaidi, and Eric Delwart

Using a simple metagenomic approach, we identified a divergent human parechovirus (HPeV) in the stool of a child in Pakistan. Genomic characterization showed this virus was distinct enough from reported HPeV types to qualify as candidate prototype for the seventh HPeV type.

Human parechoviruses (HPeVs) belong to the recently identified genus *Parechovirus* of the family *Picornaviridae*. Serologic and molecular studies show that HPeV comprises 6 neutralization serotypes or corresponding types based on capsid protein similarities, HPeV1–6. HPeV1 and HPeV2, originally known as enterovirus echoviruses 22 and 23, were isolated in 1956 (*1*). Because echoviruses 22 and 23 had serologic, molecular, and biologic properties highly distinct from other enteroviruses, they were reclassified in 1999 as members of the genus *Parechovirus* (*2*). The other 4 parechovirus types were identified more recently from young children with clinical manifestations similar to those caused by human enteroviruses: HPeV3 in 2002 (*3*), HPeV4 in 2005 (*4*), and HPeV5 (*5*) and HPeV6 (*6*) in 2006. HPeV infections occur commonly in the general population and mostly cause mild gastrointestinal and respiratory symptoms in young children (*7*). More severe consequences also have been ascribed to HPeV infections, including acute flaccid paralysis (AFP) (*3*), encephalitis (*8*), aseptic meningitis (*9*), myocarditis (*10*), neonatal sepsis (*11*), and Reye syndrome (*6*),

Nonpolio AFP may be caused by many viruses, including nonpolio enteroviruses, human adenoviruses, herpes simplex virus, Epstein-Barr virus, and West Nile virus (*12*). HPeV1 was associated with an AFP outbreak in Jamaica in 1986. In 2 of 3 AFP patients with HPeV1 detected in stool samples, antibody titer also increased significantly

(*13*). HPeV6 was isolated from the stool specimen of an AFP patient in Japan in 2001 (*6*). HPeV3 has not been reported from AFP cases but was identified in 1 transient paralysis case and believed to cause serious central nervous system symptoms more frequently than HPeV1 (*3,7*). HPeV types 2, 4, and 5 have been less often observed in clinical studies.

Using sequence-independent PCR amplification and sequence similarity searches, we recently investigated virus sequences in stool samples from children in Pakistan who had nonpolio AFP and from healthy children who had close contacts with persons who had AFP. Sequences of human parechoviruses were identified in samples from 6 of 65 persons. Analysis showed 5 HPeV infections in 56 samples from persons who had nonpolio AFP, 1 HPeV1, 1 HPeV5, and 2 HPeV6; in 1 sample, HPeV type could not be determined because the sequenced fragment was located in a phylogenetically uninformative region. A highly divergent HPeV type also was identified in 1 contact sample, and the full genome of this virus was sequenced. Phylogenetic analysis indicated that this virus, designated PAK5045, has the genetic characteristics expected of a new HPeV type.

The Study

HPeV PAK5045 was found in 1 stool sample from a healthy 2-year-old boy who had close contact with a person who had nonpolio AFP, using a previously described method applied here to stool samples (*14*). Briefly, virus nucleic acids were purified from stool samples, randomly amplified by reverse transcription (RT)–PCR using 3′ randomized RT and PCR primers, subcloned, and sequenced. HPeV sequences were abundant in 1 sample with 24/48 plasmid subclones identified as PAK5045. Assembly of these HPeV sequences produced 5 fragments covering ≈75% of the genome. Specific PCR primers were used to link these genome fragments, and rapid amplification of cDNA ends was carried out to acquire the 5′ and 3′ ends.

The nucleotide sequence of PAK5045 virus was 7,127 nt, excluding a poly (A) tail. PAK5045 contained a partial 5′ untranslated region (UTR) of 511 nt, an open reading frame (ORF) encoding a putative polyprotein precursor of 2,175 aa, and a 3′ UTR of 88 nt. The nucleotide sequence of PAK5045 was generated with at least 2× coverage except for the 5′ UTR. The full-length sequence of PAK5045 has been deposited in GenBank under accession no. EU556224.

The polyprotein of PAK5045 comprised capsid proteins VP0 (289 aa), VP3 (254 aa), and VP1 (226 aa) and nonstructural proteins 2A (149 aa), 2B (122 aa), 2C (329 aa), 3A (117 aa), 3B (20 aa), 3C (200 aa), and 3D (469 aa). Comparison of the complete ORF of PAK5045 with the 6 HPeV prototypes showed it was closely related to HPeVs and had amino acid

Table 1. Nucleotide and amino acid sequence comparisons of HPeV7 candidate prototype PAK5045 with the HPeV prototypes*

Sequence	% Nucleotide (amino acid) identity					
	HPeV1	HPeV2	HPeV3	HPeV4	HPeV5	HPeV6
5′ UTR	87.6	86.8	95.9	90.2	86.7	89.8
VP0	71.0 (76.8)	69.6 (75.8)	68.3 (72.3)	69.7 (75.1)	70.1 (75.4)	69.7 (73.7)
VP3	69.5 (75.3)	69.9 (74.1)	73.2 (83.9)	70.3 (77.5)	69.7 (74.0)	68.8 (74.0)
VP1	65.8 (69.9)	66.6 (69.5)	68.9 (77.0)	67.8 (68.6)	64.7 (66.4)	63.9 (65.0)
2A	77.9 (89.3)	75.2 (84.6)	77.5 (85.9)	80.8 (89.3)	80.5 (89.9)	76.5 (85.9)
2B	80.1 (95.9)	76.8 (95.1)	86.1 (99.2)	83.6 (99.2)	83.6 (97.5)	78.7 (96.7)
2C	78.9 (91.5)	76.5 (86.6)	83.8 (96.0)	85.9 (97.0)	80.9 (94.5)	79.5 (90.0)
3A	76.6 (88.0)	76.1 (83.8)	81.8 (94.0)	87.5 (96.6)	78.9 (82.9)	76.1 (89.7)
3B	73.3 (90.0)	75.0 (90.0)	76.7 (85.0)	85.0 (95.0)	76.7 (90.0)	70.0 (90.0)
3C	81.3 (98.0)	81.2 (98.0)	83.5 (98.0)	86.3 (98.0)	80.3 (99.0)	83.0 (99.0)
3D	83.2 (94.9)	82.8 (94.0)	88.7 (96.8)	90.8 (97.2)	83.8 (95.9)	83.6 (95.3)
3′ UTR	85.1	89.8	95.5	94.4	85.2	83.0
ORF	76.3 (86.7)	75.6 (84.8)	79.5 (89.1)	80.8 (88.5)	77.0 (86.6)	76.0 (85.4)

*Sequences for the HPeV prototypes, HPeV1 (Harris, S45208), HPeV2 (Williamson, AJ005695), HPeV3 (A308/99, AB084913), HPeV4 (K251176-02, DQ315670), HPeV5 (CT86-6760, AF055846), and HPeV6 (NII561–2000, AB252582), were obtained from GenBank. HPeV, human parechoviruses.

identity of 84.8%–89.1% and nucleotide identity of 75.6%–80.8% (Table 1). Of the 6 known HPeV types, the intertype amino acid identities ranged from 84.9% to 91.1%, and the intertype nucleotide identities of the ORF sequence ranged from 76.1% to 83.4% (data not shown), a range similar to their identities relative to PAK5045.

Phylogenetic analysis with the complete P1 amino acid sequences of fully sequenced HPeVs confirmed the existence of the 6 types defined by previous studies (Figure 1, panel A) (15). PAK5045 virus was most similar to HPeV3 strains. The identity of P1 amino acid sequences between PAK5045 virus and both HPeV3 strains analyzed was 77.5%, which
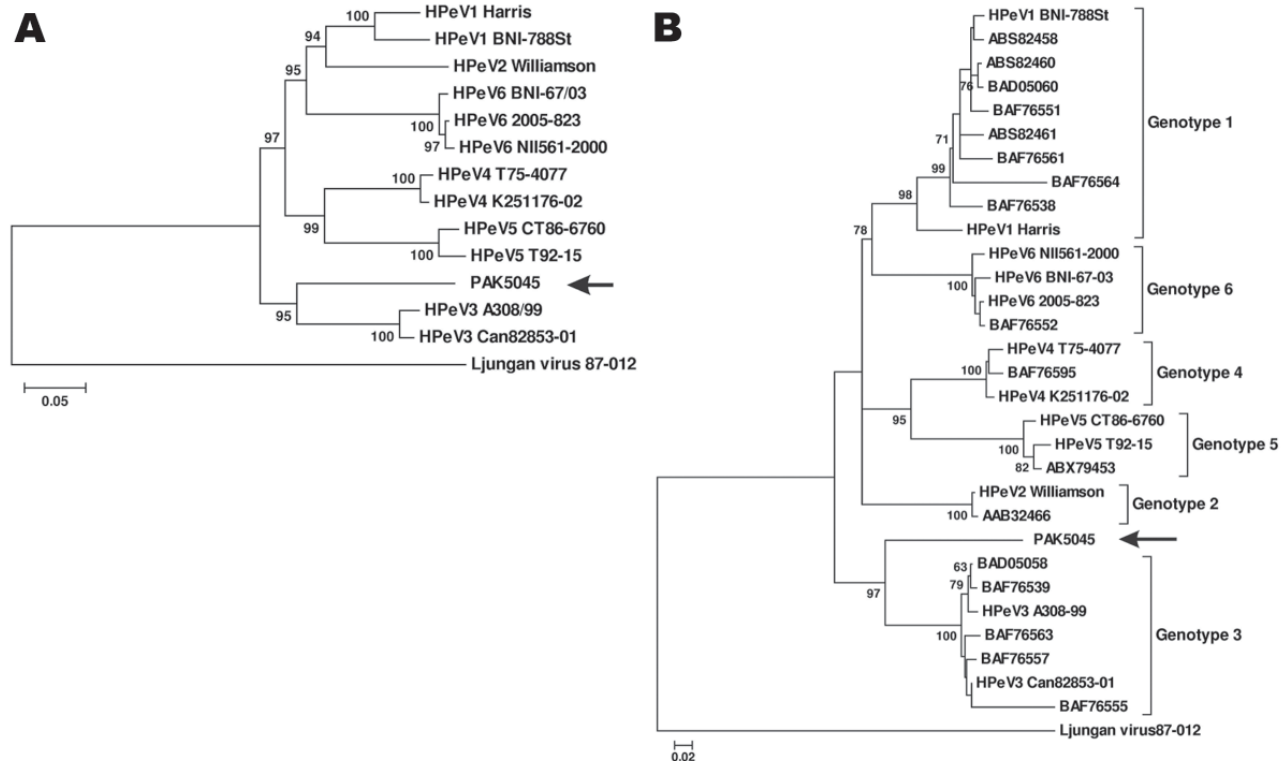

Figure 1. Phylogenetic analysis of PAK5045 virus (arrows) and parechovirus strains based on A) the complete amino acid sequence of P1 region and B) the complete amino acid sequence of viral protein 1 (VP1). All parechovirus sequences were obtained from GenBank, including 13 completely sequenced parechoviruses, human parechoviruses (HPeV) 1 Harris (Q66578), HPeV1 BNI-788St (ABK54353), HPeV2 Williamson(CAA06679), HPeV3 A308/99(BAC23086), HPeV3 Can82853-01(CAI64373),HPeV4 K251176-02 (ABC41566), HPeV4 T75-4077 (CAJ84484), HPeV5 CT86-6760 (Q9YID8), HPeV5 T92-15 (CAJ84483),HPeV6 NII561-2000 (BAF63403), HPeV6 2005-823 (ABX79460), HPeV6 BNI-67/03 (ABS82455), Ljungan virus 87-012 (AAM46079), and 17 sequences with accession numbers shown directly on the tree. Both trees were constructed by the neighbor-joining method with 1,000 bootstrap replicates using MEGA4.

was lower than some HPeVs intertype amino acid identities (e.g., average of 82.4% between HPeV1 and HPeV2, 78.3% between HPeV1 and HPeV4, 80.4% between HPeV1 and HPeV6, 78.4% between HPeV2 and HPeV6, and 80.5% between HPeV4 and HPeV5) (data not shown).

Consistent with the P1 amino acid tree, phylogenetic analysis with VP1 capsid proteins also showed 6 established types (Figure 1, panel B) (5). PAK5045 VP1 was slightly closer to type 3 strains, with the greatest amino acid identity being 78.8% (Table 2), and more divergent from the other established HPeV types. We retrieved from GenBank, and then analyzed, genetic relationships among 92 full-length VP1 amino acid sequences and with PAK5045. None clustered with PAK5045 as a close genetic lineage. The amino acid identities between PAK5045 and HPeV3 strains ranged from 69.9% to 78.8%, outside the HPeV3 intratype range of 85.8%–100% (Table 2).

The PAK5045 polyprotein contained 9 putative cleavage sites at VP0/VP3 (T/A), VP3/VP1 (Q/N), VP1/2A (E/S), 2A/2B (Q/G), 2B/2C (Q/G), 2C/3A (Q/T), 3A/3B (E/R), 3B/3C(Q/R), and 3C/3D (Q/G). Alignments showed that VP3/VP1, VP1/2A, and 2C/3A cleavage sites differed for PAK5045 relative to those of fully sequenced HPeVs strains, whereas the other 6 sites were conserved. The cleavage site in VP0/VP3 of PAK5045 was identical to that of HPeV2 but not to those of other types. The VP1/2A cleavage site was identical between PAK5045 and HPeV3 strains A308/99 and Can82853-01 but not other HPeVs. The RGD motif (arginine-glycine-aspartic acid) at the C terminus of VP1 was absent in PAK5045 and in HPeV3 strains A308/99 and Can82853-01, which indicates that mechanisms other than RGD binding to integrins may occur during PAK5045 infection.

To identify recombination events between the different HPeV types, we performed SimPlot analysis (http://sray.med.som.jhmi.edu/SCRoftware/simplot) of the 6 complete nucleotide HPeV prototype genomes against PAK5045 (Figure 2). In general, PAK5045 was closer to HPeV3 and HPeV4 than to the other viruses. PAK5045 showed a relatively higher degree of nucleotide similarity to HPeV3 A308/99 in the P1 region consistent with the P1 phylogenetic tree. Downstream of nucleotide position 3600, HPeV4 K251176-02 became the closest relative of PAK5045 in most of the nonstructural (P2/3) region, which suggests an ancient recombination event.
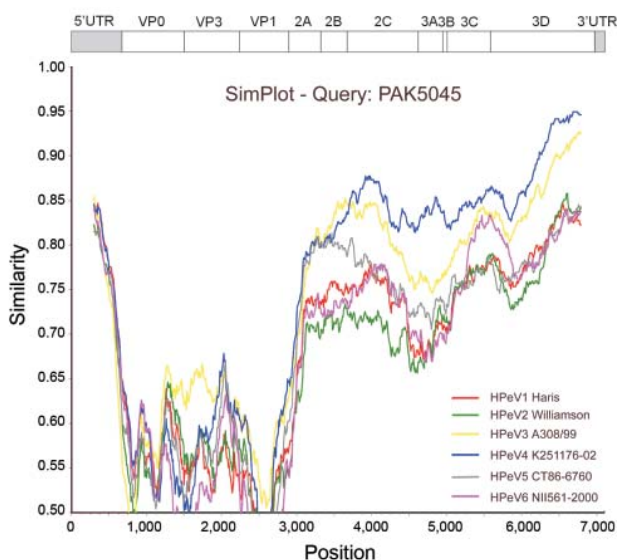

Figure 2. SimPlot (http://sray.med.som.jhmi.edu/SCRoftware/simplot) analysis of the full-length sequences of the human parechovirus (HPeV) prototypes against PAK5045 query, based on nucleotide similarities. Each curve compares the PAK5045 genome with an HPeV prototype. The Kimura 2-parameter model was applied with a transition/transversion (Ts/Tv) ratio of 3.0 (5), and a sliding window of 600 nt with a step size of 10 nt was used.

Table 2. VP1 amino acid sequence comparisons of HPeV types*

Type	Average (range) of VP1 amino acid identities, %						
	HPeV1	HPeV2	HPeV3	HPeV4	HPeV5	HPeV6	PAK5045
HPeV1	94.7 (81.8–100.0)	78.3 (71.7–80.0)	71.2 (61.9–73.9)	75.6 (69.7–78.4)	72.6 (66.7–74.9)	78.2 (71.9–81.8)	68.1 (64.6–69.9)
HPeV2		99.4 (99.1–100.0)	71.4 (65.5–72.6)	74.9 (74.3–76.1)	71.8 (70.4–72.6)	73.7 (73.5–73.9)	69.5 (–)
HPeV3			97.3 (85.8–100.0)	70.8 (66.4–71.7)	66.8 (60.6–68.6)	73.9 (66.8–75.7)	77.6 (69.9–78.8)
HPeV4				97.0 (96.6–97.8)	78.3 (75.9–80.2)	72.8 (72.3–73.2)	68.4 (68.1–68.6)
HPeV5					96.9 (94.8–100.0)	72.0 (71.0–72.7)	66.1 (65.5–66.4)
HPeV6						97.2 (95.7–98.7)	64.9 (64.6–65.0)
PAK5045							–

*92 full-length VP1 amino acid sequences were obtained from GenBank, including 46 HPeV1, 4 HPeV2, 31 HPeV3, 3 HPeV4, 4 HPeV5, and 4 HPeV 6 VP1 amino acid sequences. The accession nos. are AAA72291, AAB32466, AAB23363, AAC79756, ABC41566, ABK54353, ABS82455, ABS82457–82462, ABX79460, ABX79453, BAC23086, BAD05057–05062, BAF63403, BAF76536–76596, CAA06679, CAI64373, CAJ84483–84484, NP_046804, NP_740386, Q66578, and Q9YID8. HPeV, human parechoviruses; VP1, viral protein 1.

Conclusions

We identified and characterized a novel HPeV type from the stool sample of a healthy child who had been in close contact with a person who had nonpolio AFP. The genome sequence diverged sufficiently from the 6 known HPeVs to qualify as a candidate for the prototype of HPeV7. Using only a low-level shotgun sequencing method, we detected HPeVs in 9% (6/65) of stool samples from patients with nonpolio AFP, including HPeV types 1, 5, 6, and 7. A more sensitive method, such as HPeV-directed RT-nested PCR, is likely to have detected a higher prevalence. The median age of sampled patients was 3 years (range 1 month–15 years), and all HPeV-positive patients were <3 years of age.

In a previous study, HPeVs were isolated from 0.3% of 13,656 various clinical samples collected in Japan (14 HPeV1, 16 HPeV3, 10 HPeV6, and 1 HPeV4) (6). In Germany, the detection rate of HPeVs did not differ significantly between patients with acute diarrhea and controls, with 11.6% (7/60) of children <2 years of age being HPeV positive (15). In a Dutch study of 303 isolates showing cytopathic effects consistent with enterovirus infection, 12% were HPeV positive, with 27 HPeV1 and 10 HPeV3, all in children <3 years of age (7). HPeV infection, therefore, seems to be associated with young children (<3 years). More studies are needed to associate HPeV infection (with any genotypes) with development of neurologic disease, such as AFP.

Dr Li is a research fellow at the Blood Systems Research Institute, San Francisco. Her current research interest focuses on discovery of novel viruses using metagenomic methods.

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Clinical Relevance of Nontuberculous Mycobacteria, Oman

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Little is known about the clinical relevance of nontuberculous mycobacteria (NTM) in the Arabian Peninsula. We assessed the prevalence and studied a random sample of isolates at a reference laboratory in Muscat, Oman. NTM cause disease in this region, and their prevalence has increased.

Nontuberculous mycobacteria (NTM) are common inhabitants of the environment and have been cultured from water, soil, and animal sources worldwide. NTM are opportunistic pathogens, mostly affecting patients with preexisting pulmonary disease such as chronic obstructive pulmonary disease or tuberculosis (TB), or those with systemic impairment of immunity. The latter group includes those with HIV infection, those using immunosuppressive drugs, and those with leukemia (1).

Because NTM are common in the environment and resistant to commonly used disinfectants, they can be present in nonsterile patient material such as sputum and contaminated medical equipment (bronchoscope washers or samples in the laboratory) and consequently cause pseudoinfection (1,2). To distinguish pseudoinfections from NTM disease and establish the clinical relevance of isolated NTM, the American Thoracic Society (ATS) has published diagnostic criteria (1).

Studies on the clinical relevance of NTM have traditionally been restricted to western countries, where the incidence of TB is relatively low. More recently, research has been initiated in African and East Asian countries (1–5). There have been no recent reports on isolation and clinical relevance of NTM on the Arabian Peninsula. We analyzed a random sample of NTM isolated from clinical samples

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in Oman by using molecular techniques. Their clinical relevance was retrospectively analyzed by applying the updated ATS diagnostic criteria for NTM disease (1).

The Study

Prevalence of NTM at the Central Public Health Laboratory (CPHL), the national TB reference laboratory of the Ministry of Health in Muscat, Oman, was assessed by using a laboratory database. The CPHL Institutional Review Board reviewed and approved this study. The CPHL received 5,488 samples submitted for *Mycobacterium* spp. culture during 2006–2007. Samples were subcultured in Lowenstein-Jensen medium and automated Mycobacterial Growth Indicator Tubes (MGIT960; Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 36°C. A total of 491 (9%) samples yielded positive cultures. *M. tuberculosis* complex bacteria were isolated from 445 (91%) samples, and NTM were isolated from 46 samples (9%). Most NTM were cultured from respiratory samples (sputum, n = 36, 78%; bronchial lavage, n = 2, 4%); the remainder were from lymph nodes (n = 3, 6%), urine (n = 2, 4%), or other sources (n = 3, 6%). The percentage of NTM increased from 7.6% (18/235) in 2006 to 10.9% (28/256) in 2007.

Thirteen samples were randomly selected from all NTM isolated at CPHL during January 2006–September 2007. Selected NTM were subjected to molecular identification at the Dutch National Mycobacteria Reference Laboratory (National Institute for Public Health and the Environment, Bilthoven, the Netherlands). To identify NTM, after eliminating isolates of the *M. tuberculosis* complex by using the GenoType MTBC assay (Hain Lifesciences, Nehren, Germany), we used the Inno-Lipa Mycobacteria version 2 reverse line blot (Innogenetics, Ghent, Belgium). Both assays were used according to the manufacturer's instructions. If no species-specific result was obtained, additional sequencing of the hypervariable region A of the 16S rDNA gene was performed (6).

The 13 samples were identified as 9 *M. avium* complex (MAC; 3 *M. intracellulare* sequevars, 3 *M. chimaera*, 1 *M. colombiense*, 1 *M. avium*, and 1 untyped), 1 *M. marinum*, 1 *M. kansasii*, 1 *M. simiae*, and 1 *M. flavescens* (Table). One sample could not be identified beyond the *M. avium–intracellulare–scrofulaceum* complex level because insufficient DNA was available for further analyses. Because our molecular identification method does not distinguish *M. marinum* from *M. ulcerans*, we performed a light exposure test, which identified yellow colony pigmentation typical for *M. marinum*. One sample yielded *M. tuberculosis* and *M. intracellulare* (Table).

We assessed the clinical relevance of isolates from 13 patients in a retrospective study by applying ATS diagnostic criteria and scoring demographic and clinical data. Results are detailed in the Table. Seven (54%) of the patients

Table. Clinical and microbiologic data for 13 patients with *Mycobacterium* spp. infections, Oman, 2006–2007*

Patient no./sex/age, y	Species	AFB smear	Positive cultures	Source	Condition	Symp.	Chest radiograph	2007 ATS criteria	Therapy	Outcome
1/F/64	*M. intracellulare*†	+	Multiple	Sputum	–	PC, F, WL,CP, M	NP	Met	HRZE	Improved
2/M/29	MAIS complex	+	Single	Sputum	–	PC, Hp, WL	Cavities	Met	HRZE	Improved
3/F/31	*M. chimaera*‡	–	Multiple	Sputum	–	None	RUL bronch.	Not met	None	Stable
4/M/28	*M. chimaera*‡	+	Multiple	Sputum	–	PC, WL	RUL multiple scars, left lung destroyed, abscess, PT	Met	SClaCip	Failure
5/M/18	16S: *M. colombiense*	–	Single	BAL	HD	PC, Hp, CP	NP	Met	HRE	Improved
6/M/57	16S: *M. flavescens*	NP	Single	Urine	–	AP	NP	Not met	NA	NA
7/F/57	*M. simiae*	–	Single	Sputum	HIV	PC	Patchy opacities in LUL and lingula	Met	HRE	Improved
8/F/12	*M. kansasii* III/IV/V	–	Single	Sputum	–	None	Normal	Not met	NA	NA
9/M/43	*M. tuberculosis* and *M. intracellulare*†	+	Multiple	Sputum	–	PC, F, WL, M	Cavities	NA	HRZES	Failure
10/F/7	*M. marinum*	–	Single	Skin	_	Skin lesion	NP	Met	ECla	Improved
11/F/65	*M. chimaera*‡	+	Multiple	Sputum	–	PC, BA	Destroyed left lung	Met	ClaCip	Improved
12/F/83	*M. avium*	–	Single	Sputum	–	None	RUL infiltration	Not met	NA	NA
13/M/63	*M. intracellulare*†	+	Single	Sputum	Prior PNTM disease	PC, WL, M	Bronch., PI	Met	RECip	Improved

*AFB, acid-fast bacilli; Symp., symptoms; ATS, American Thoracic Society; PC, productive cough; F, fever; WL, weight loss; CP, chest pain; M, malaise/fatigue; NP, not performed; H, isoniazid; R, rifampicin; Z, pyrazinamide; E, ethambutol; MAIS, *M. avium–intracellulare–scrofulaceum*; Hp, hemoptysis; RUL, right upper lobe of lung; LUL, left upper lobe of lung; Bronch., bronchiectasis; PT, pleural thickening; S, streptomycin; Cla, clarithromycin; Cip, ciprofloxacin; 16S, identified by 16S rDNA gene sequencing; BAL, bronchoalveolar lavage fluid; HD, heart disease; AP, abdominal pain; NA, not applicable; BA, backache; PI, parenchymal infiltration; PNTM, pulmonary nontuberculous mycobacteria.
†Reaction with the MIN-1 probe; *M. intracellulare* sequevar Min-A, -B, -C, or -D.
‡Reaction with the MIN-2 probe; *M. intracellulare* sequevar MAC-A, which was recently elevated to the species level (*M. chimaera*) (7).

were female (mean age 43 years). Eight (62%) of 13 patients met the ATS diagnostic criteria and were thus likely to have NTM disease. Among 9 patients with MAC isolates, 6 (67%) had MAC disease. Most (11, 85%) isolates were cultured from pulmonary samples. Fibrocavitary and nodular-bronchiectatic pulmonary NTM disease were noted, with a predominance of fibrocavitary disease (Table).

Information on predisposing conditions was not available for most patients, although a destroyed lung on chest radiographs suggested previous pulmonary disease. One patient was HIV positive, and another patient had a relapse of pulmonary NTM disease.

Eight patients began treatment, mostly with first-line treatment for TB. Three patients with pulmonary MAC disease and 1 with *M. marinum* skin disease received regimens that included macrolides, fluoroquinolones, or both. Therapy resulted in clinical improvement in all but 1 patient. Most patients are still receiving treatment.

Conclusions

A total of 9% all *Mycobacterium*-positive cultures at CPHL in Muscat, Oman, yielded NTM. Although this conclusion is based on limited data, the prevalence of NTM seems to be increasing in Oman. Few studies are available, but this increase may be true for the entire Middle East region.

Eight of 13 patients met the ATS diagnostic criteria; this finding probably reflects a selection bias because CPHL is a reference laboratory. Nevertheless, these findings indicate that NTM in Oman and throughout the Middle East region is a serious issue that requires attention by clinicians and microbiologists.

Most isolates were MAC members (9/13, 69%), a predominance also noted in previous studies in the United States, Europe, and west Asian countries (25%) (*1,8*), and South Korea (48%) (*9*). MAC isolates from Oman were mostly *M. intracellulare* sequevars. Infrequent isolation of *M. avium* is noteworthy, despite the small number of

isolates in the random sample. Previous North American studies have suggested that *M. intracellulare* is the more common pulmonary pathogen within the MAC (*1*). We identified 3 pulmonary samples as MAC-A strains, which were recently elevated to species level as *M. chimaera* (*7*). Two samples were clinically relevant. Although *M. chimaera* has been assumed to be highly virulent (*7*), a recent study in Germany found only 3.3% of 90 *M. chimaera* isolates to be clinically relevant (*10*).

M. colombiense was first described as a causative agent of mostly disseminated disease in HIV patients from Colombia (*11*) and was recently isolated from a child with lymphadenopathy in Spain (*12*). Its isolation in other countries and from respiratory samples in HIV-negative patients has not been reported. Isolation of *M. simiae* is a serious concern because this species has been reported to be prevalent in the Middle East (*1*), and HIV-associated disease has been reported in the region (*13*).

Most patients in our study received standard treatment for TB. Although treatment should be prolonged and pyrazinamide discontinued because of natural resistance to pyrazinamide in NTM, the choice of first-line treatment for TB, without companion drugs such as macrolides or fluoroquinolones, is supported by a recently published trial of the British Thoracic Society (*14*).

In summary, NTM are a serious issue in Oman and their prevalence may be increasing. Our random sample demonstrates that MAC isolates are most frequently isolated. True NTM disease, on the basis of ATS diagnostic criteria, was diagnosed in 62% of the patients assessed. Isolation of NTM is clinically relevant on the Arabian Peninsula and warrants further study.

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Dr Al-Mahruqi is a staff member of the Central Public Health Laboratory in Muscat, Oman. Her research interests are the molecular epidemiology of TB and the clinical relevance of isolation of nontuberculous mycobacteria in Oman.

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Enteroviruses in Patients with Acute Encephalitis, Uttar Pradesh, India

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An outbreak of viral encephalitis occurred in northern India in 2006. Attempts to identify an etiologic agent in cerebrospinal fluid by using reverse transcription–PCR showed positivity to enterovirus (EV) in 66 (21.6%) of 306 patients. Sequencing and phylogenetic analyses of PCR products from 59 (89.3%) of 66 specimens showed similarity with EV-89 and EV-76 sequences.

Acute viral encephalitis is caused by a wide range of viruses and can occur either in sporadic episodes or in outbreaks. Viral etiologic agents that have been identified as causing encephalitis include herpesvirus, enterovirus, alphavirus, influenza A virus, rabies virus, HIV, flavivirus, and Chandipura (CHP) virus (1,2). An outbreak of viral encephalitis was reported from April through October 2006 from predominantly Gorakhpur and 5 adjoining districts of eastern Uttar Pradesh (Maharajganj, Kushinagar, Sant Kabir Nagar, Siddharthnagar, and Deoria) and 2 adjoining districts of Bihar (Gopalganj and West Champaran), locations where Japanese encephalitis (JE) is known to be endemic in India. According to state government health services records, 1,912 cases of viral encephalitis occurred in these areas, and 411 (21.5%) patients died. From August through September 2006, we investigated 306 patients admitted with encephalitis to Baba Raghav Das Medical College in Gorakhpur, Uttar Pradesh. The patients represented all 8 districts of eastern Uttar Pradesh. ELISA and reverse transcription–PCR (RT-PCR) performed on the patients' cerebrospinal fluid (CSF) samples identified 40 (13.1%) of 306 specimens as positive for JE virus (3). Laboratory tests

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were negative for alphavirus and CHP virus, and the etiologic agent in a large number of cases was unidentified.

Enteroviruses (EVs) cause a wide variety of diseases that range from nonspecific viral illness to mild infections of herpangina and hand, foot, and mouth disease to potentially serious diseases such as myopericarditis, meningitis, myelitis, and neonatal sepsis. EVs are also etiologic agents of encephalitis outbreaks in humans (4). These viruses comprise more than 90 serotypes, and most are known to cause human infections. We focused on the detection, isolation, and molecular characterization of EVs in 306 patients from eastern Uttar Pradesh.

The Study

A total of 850 specimens collected from 306 patients who had encephalitis included 306 CSF specimens, 304 blood samples, 120 throat swabs, and 120 rectal swabs. All samples were stored at –20°C before being transported for analysis and thereafter were stored at –70°C at the National Institute of Virology in Pune, India. Laboratory tests conducted by state government health services of Uttar Pradesh were negative for bacteria and malaria. According to standard protocol (2), virus isolation was attempted in human rhabdosarcoma (RD) and in baby hamster kidney (BHK) cell lines.

Separate aliquots were processed in 2 laboratories to maintain quality control and monitor possible contamination during PCR processing. Viral nucleic acids were extracted by using viral RNA mini kits (QIAamp, Qiagen, Hilden, Germany). RT-PCR was performed for EV by using 5′ noncoding region (NCR)–specific primers, as has been described (5,6). Genotyping was conducted by using RT-PCR of virion protein (VP) 1/2A and VP1 regions and sequencing (7,8). Table 1 describes the locations and sequences of the primers used in the assays.

PCR products were purified by using a Gel Extraction Kit (QIAquick, Qiagen). Both strands were sequenced by using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Carlsbad, CA, USA) in ABI PRISM 3130 XL Genetic Analyser (Applied Biosystems). MEGA 3.1 software generated the phylogenetic tree by using the neighbor-joining algorithm and Kimura 2–parameter distance model and applying a bootstrap test that used 1,000 bootstrap replications (9).

Patient age ranged from <1 month to 15 years. Clinical histories available for 253 of the 306 patients showed fever and altered sensorium in 100.0%, hepatomegaly in 70 (27.8%), splenomegaly in 49 (19.4%), and meningeal signs in 35 (13.9%) of the 253 patients.

Specimens available in sufficient quantity were inoculated into RD and BHK cell lines. Specimens that were adequate for isolation included 85 of 306 CSF specimens, 18 of 304 serum samples, 19 of 120 rectal swabs, and 19

Table 1. Primers used for PCR and sequencing for enterovirus isolates from encephalitis patients, Uttar Pradesh, India, 2006*

Serial no.	Region	Location	Primer sequence (5′ → 3′)	Product size, bp
1	5' NCR	64–84	CGGTACCTTTGTACGCCTGT	537
2	5' NCR	601–582	ATTGTCACCATAAGCAGCCA	537
3	5' NCR	166–186	CAAGCACTTCTGTTTCCCCGG	400
4	5' NCR	566–546	GAAACACGGACACCCAAAGTA	400
5	VP1/2A	EV-012 (2917–2936)	ATGTAYGTICCICCIGGIGG	457
6	VP1/2A	EV-040 (2917–2936)	ATGTAYRTICCIMCIGGIGC	457
7	VP1/2A	EV-011 (3374–3355)	GCICCIGAYTGITGICCRAA	457
8	VP1 cDNA	AN32 (3009–3002)	GTYTGCCA	NA
9	VP1 cDNA	AN33 (3009–3002)	GAYTGCCA	NA
10	VP1 cDNA	AN34 (3111–3104)	CCRTCRTA	NA
11	VP1 cDNA	AN35 (3009–3002)	RCTYTGCCA	NA
12	VP3	224(1977–1996)	GCIATGYTIGGIACICAYRT	762
13	VP1	222 (2969–2951)	CICCIGGIGGIAYRWACAT	762
14	VP1	AN89 (2602–2627)	CCAGCACTGACAGCAGYNGARAYNGG	348–393
15	VP1	AN88 (2977–2951)	TACTGGACCACCTGGNGGNAYRWACAT	348–393

*NCR, noncoding region of viral genome; VP, virion protein; NA, not applicable.

of 120 throat swabs. Cytopathic effect was observed in cell cultures inoculated with 4 CSF specimens, 2 rectal swabs, 2 throat swabs, and 1 serum sample. Electron microscopic examination of cultures infected with 2 CSF samples showed picornavirus-like particles 25–27 nm in diameter. Attempts to detect EV RNA in the isolates and clinical specimens used nested RT-PCR in 5′ NCR. Eight of 9 cultures showed amplicons of 407 bp. Sequences of amplicons from 3 CSF specimens and 2 rectal swabs showed 97.2%–98.9% homology with EV-89 (i.e., the strain named BANoo-10359, GenBank accession no. AY697459) and 95.7%–96.9% homology with EV-76 (FRA91-10369, GenBank accession no. AY697458). Sequences from 1 isolate from a CSF specimen and 1 isolate from a rectal swab showed 100.0% homology with coxsackie virus B3 (CV-B3) strain 20. One isolate from serum showed 98.3% homology with coxsackie virus B1 (CV-B1) strain SAMP2.17.

Sixty-six (21.5%) of 306 CSF specimens, 7 (6.4%) of 110 rectal swabs, 4 (3.7%) of 110 throat swabs, and 1 (5.5%) of 18 serum samples showed amplification in 5′ NCR of the EV genome. Sequences of 64 of 78 (82.1%) PCR products (59 from CSF specimens, 4 from rectal swabs, and 1 from a throat swab) showed 97.2%–98.9% and 95.7%–96.9% homology with EV-89 and EV-76, respectively. Ten (12.8%) products (7 from CSF, 2 from rectal swabs, and 1 from serum) showed 99.3%–100.0% homology with CV-B3 (Figure 1). Three PCR products, each derived from a throat swab, showed 93.3%–96.6% homology with coxsackie virus A (CV-A), echovirus 11, and echovirus 30, respectively. PCR products from a rectal swab showed 96.3% homology with CV-B1. Multiple specimen positivity was noted in 6 patients who tested positive for EV RNA.

Isolates from 2 of 5 cell cultures, 2 of 59 CSF specimens, and 1 of 4 rectal swabs contained EV-76. Two of 4 rectal swabs were characterized as EV-89 on the basis of

partial VP1/2A (2917–3374) or VP1 (2602–2977) gene sequences. Phylogenetic analysis revealed 92.7%–97.7% homology with Bangladesh EV-76 strains (GenBank accession nos. AY697463, AY697464, AY697471, AY697469, AY697462, and AY697468) and 93.6%–94.5% homology with EV-89 strain (GenBank accession no. AY697459) (Figure 2). Within EV-76 and EV-89 strains of the study, homology ranged from 81.2% to 91.3%. Attempts to amplify VP1/2A or VP1 regions of EV RNA detected in most clinical specimens failed despite the use of sensitive primer pairs that have been discussed recently (10).

Table 2 describes details of clinical findings in the subsets of EV-positive and EV-negative specimens of the patients for whom clinical histories were available. Further, hepatomegaly and splenomegaly appeared to be proportionately higher in patients with enteroviral infections than in patients whose specimens were negative for EV and JE virus.

Conclusions

The viral RNA detected in CSF samples from patients hospitalized with encephalitis in Uttar Pradesh showed close identity with the EV-89 and EV-76 that recently were reported as an unusual group classified genetically as group A EV (EV-A) (10). Presence of the virus was also confirmed by its isolation and typing. Human EV-76 was detected in isolates in 1 rectal swab and 2 CSF specimens, and human EV-89 was detected in 2 rectal swabs by using amplification of VP1/2A or VP1 regions. Sequence analysis showed nt homology of 92.7%–97.7% with Bangladesh EV-76 and EV-89 strains recovered from patients with acute flaccid paralysis (AFP). The failure of amplification of typing regions in most specimens may be due to a low viral load.

EVs are known to cause severe neurologic diseases ranging from AFP to encephalitis (11). In recent years,

Southeast Asian countries have reported outbreaks of encephalitis caused by EV-71 (*12,13*). During AFP surveillance activities, Bangladesh strains were isolated from stool specimens (*14*). AFP patients infected with echoviruses and coxsackie B viruses also have been detected in India (*15*). Isolation of EV from clinical specimens collected from children with encephalitis in the present study indicates viable virus. Detection of EV-89/76 RNA in the CSF of ≈20% of the patients suggests the association of these viruses with encephalitis. Also, in 10 (3.3%) of 306 patients, co-infections of JE virus and EV were detected. Further studies are needed to understand the relative contributions of these viruses in causing sporadic and outbreak infections of encephalitis.
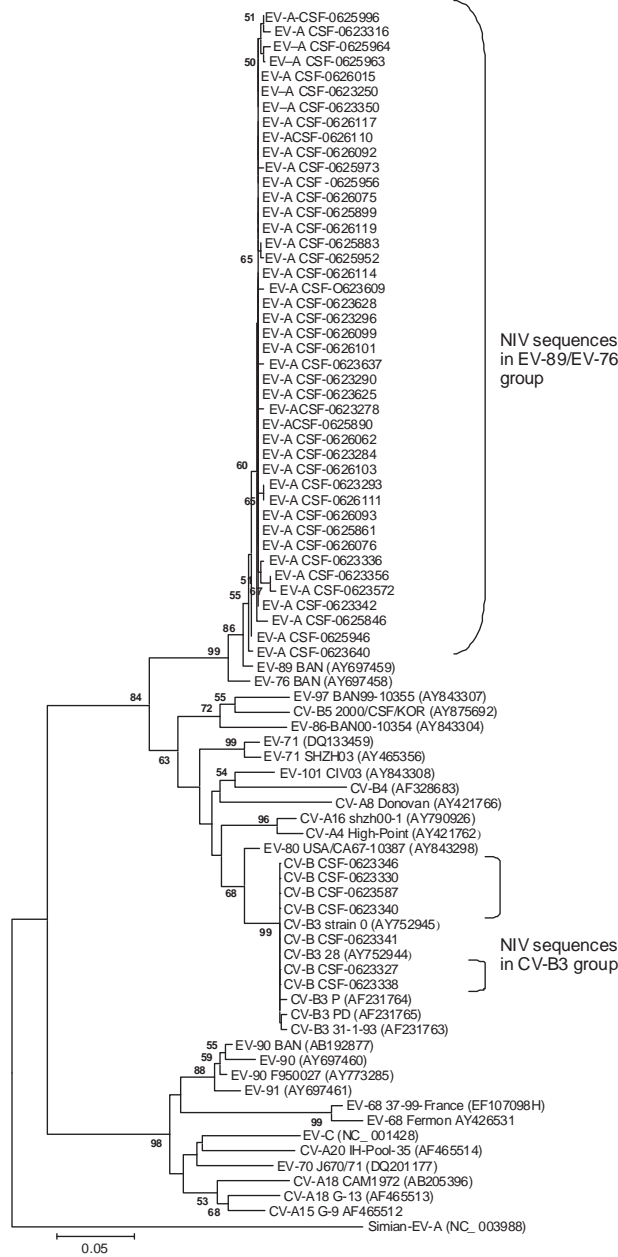
Figure 1. Phylogenetic tree based on partial 5' noncoding region sequences of enterovirus (EV) genome detected in cerebrospinal fluid samples from encephalitis patients. Specimens are identified by repository serial numbers obtained from the National Institute of Virology (NIV), Pune, India. GenBank accession nos. EU672893–EU762967 indicate the nucleotide sequences of EV strains of the present study. Scale bar indicates nucleotide substitutions per site. EV, enterovirus; CSF, cerebrospinal fluid; CV-A, coxsackie virus A; CV-B, coxsackie virus B; HEV, human enterovirus.
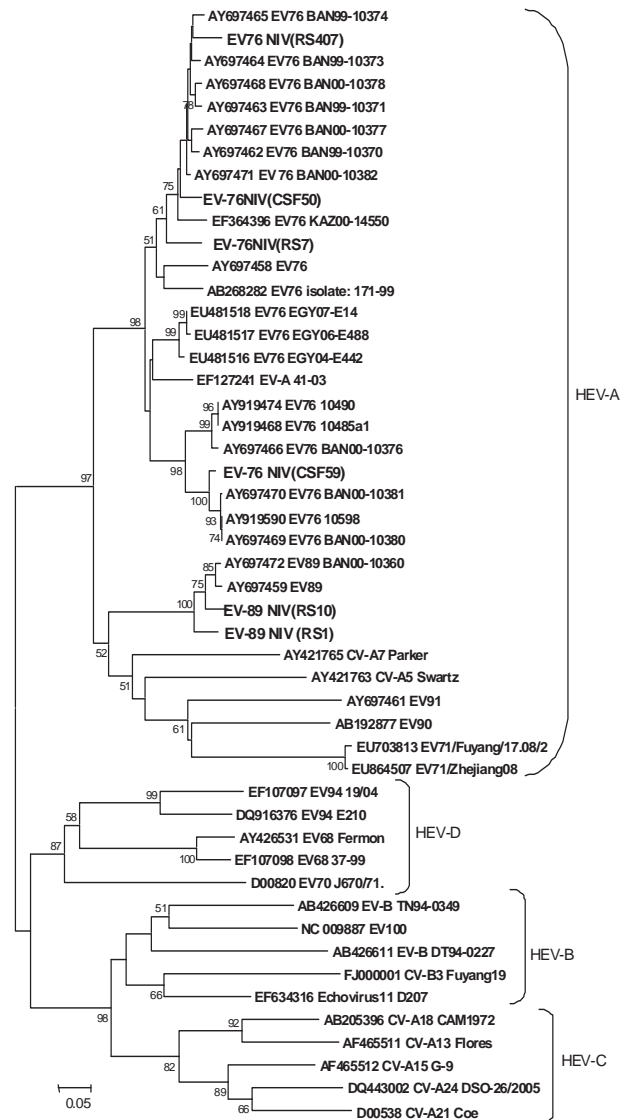

Figure 2. Phylogenetic tree based on partial virion protein 1 (VP1) sequences (2602–2977) detected in enterovirus (EV) isolates and clinical specimens from encephalitis patients. GenBank accession nos. indicate the nucleotide sequences of EV strains of the present study. Scale bar indicates nucleotide substitutions per site. EV, enterovirus; CV-A, coxsackie virus A; CV-B, coxsackie virus B; HEV, human enterovirus; NIV, National Institute of Virology, Pune, India.

Table 2. Clinical features of encephalitis patients with and without EV infections, Upper Pradesh, India, 2006*

Feature	No. (%) EV positive	No. (%) EV negative
Fever	51 (100.0)	202 (100.0)
Altered sensorium	51 (100.0)	202 (100.0)
Hepatomegaly	13 (25.49)	21 (10.3)
Splenomegaly	13 (25.49)	32 (15.8)
Brisk DTR	4 (8.51)†	21 (10.3)
Meningeal signs	6 (11.7)	20 (9.9)
Total no. of patients	51	202

*EV, enterovirus; DTR, deep tendon reflex.
†Denominator is 47 because these data were unavailable for 4 patients.

Accumulation of water in a saucer-shaped landscape (*terai*) and extensive rice cultivation in eastern Uttar Pradesh and adjoining regions favor the growth of vector mosquito populations and waterborne pathogens. Though the source of infection in the present study is unclear, the data warrant active surveillance of encephalitis cases. Inadequate hygiene and the unsanitary conditions that prevail in the study region may encourage the spread of EV infections in the community. Studies conducted on environmental samples may provide clues related to the dynamics of EV infections in humans.

Acknowledgements

Mr Sapkal is a research scientist working in the Japanese encephalitis group at the National Institute of Virology, Pune, India. His research interests include diagnostic virology of Japanese encephalitis and West Nile viruses, molecular pathogenesis, and emerging viral infections. He has worked on isolation of Japanese encephalitis, dengue, and Chandipura viruses in various epidemics using peripheral blood mononuclear cells coculture systems.

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Peste des Petits Ruminants Virus in Tibet, China

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Serologic and molecular evidence indicates that peste des petits ruminants virus (PPRV) infection has emerged in goats and sheep in the Ngari region of southwestern Tibet, People's Republic of China. Phylogenetic analysis confirms that the PPRV strain from Tibet is classified as lineage 4 and is closely related to viruses currently circulating in neighboring countries of southern Asia.

P este des petits ruminants (PPR) is an acute and highly contagious viral disease contracted by small ruminants such as goats and sheep and causing high rates of illness and death. The disease is endemic in parts of sub-Saharan Africa, the Middle East, and Asia. The PPR virus (PPRV) genogroup consists of 4 lineages (*1,2*). PPRV infection was officially reported in the Ngari region of western Tibet, People's Republic of China, in July 2007. Our study assesses the prevalence of PPRV infection in goats and sheep by region in Tibet. We also characterize strains of the virus by comparing part of the genome sequences with other PPRV sequences available in the GenBank database.

The Study

Small ruminants in regions throughout Tibet were examined for PPRV antibody from July 2007 through November 2007. The sampling procedure focused on 3 groups of animals. The first comprised 718 animals in 4 counties (Rutog, Ge'gyai, Gerze, and Zada) in the Ngari region, where animals having clinical signs of PPRV infection had been reported by local authorities. The second group included 298 animals in Gar and Bulang counties in the same region and in 2 counties bordering the Ngari region (Nyima in Nagqu region and Zhongba in Shigatse region). The third group contained 520 animals in 5 counties within 3 separate regions (Nyalam and Yadong in Shigatse region, Cona and Lhozhag in Shannan region, and Zayu in Nyingchi region).

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Table 1. PPRV antibody in animals sampled in Tibet, China, 2007*

Region	County	No. samples	No. (%) PPRV positive
Ngari	Gerze	131	59 (45.0)
	Ge'gyai	314	90 (28.7)
	Rutog	209	122 (58.4)
	Zada	64	0
	Gar	50	0
	Bulang	68	0
Nyingchi	Zayu	60	0
Nagqu	Nyima	60	0
Shigatse	Nyalam	120	0
	Yadong	66	0
	Zhongba	120	0
Shannan	Cona	135	0
	Lhozhag	139	0
Total		1,536	271 (17.6)

*PPRV, peste des petits ruminants virus.

We examined 1,536 animals (771 goats and 765 sheep) and collected serum samples from each. A competitive ELISA that used a monoclonal antibody to the N protein (*3*) identified 271 animals (17.6%) having antibody to PPRV. The PPRV-positive sera were collected from Rutog (122/209), Gerze (59/131), and Ge'gyai (90/314) counties in the Ngari region (Table 1). Rates of PPRV infection were higher in goats than in sheep. Of 763 goats examined in the Ngari region, 263 (34.5%) were seropositive for PPRV. The highest seroprevalence (61.6%, 121/198) was found in goats in Rutog County. Only 8 (11%) of 73 sheep examined in the Ngari region were seropositive for PPRV (Table 2).

Field samples, including organ (lymph node, spleen, lung, and intestine) and swab specimens, were obtained from 49 goats and sheep suspected of being infected with PPRV. These animals inhabited 4 counties in the Ngari region (Ge'gyai n = 33, Zada n = 7, Gerze n = 5, and Rutog n = 4). Two reverse transcription–PCRs (RT-PCR) and 1 newly developed and validated real-time quantitative RT-PCR (qRT-PCR) were conducted to determine whether the animals had viral RNA (*4–6*). The first RT-PCR (N RT-PCR), which amplified a 351-bp fragment in the N protein gene, detected virus in 28 samples. The second RT-PCR (F RT-PCR), which amplified a 448-bp fragment in the F protein gene, detected virus in 27 samples. The qRT-PCR detected virus in 37 samples. Use of qRT-PCR and 1 of the 2 RT-PCRs showed that 31 animals were found to contain viral RNA. In goats, 23 (77%) of the 30 samples contained viral RNA, and 2 (29%) of 7 sheep samples contained viral RNA. Most (61%) infected animals showed a high viral load with individual cycle threshold (Ct) values <30. Almost one third (29%) had a moderate viral load (Ct 30–35), and 10% had a Ct value >35. The distribution of Ct values differed slightly according to the infected animal's origin. All animals from Gerze County had low Ct values (Ct 19–

Table 2. Antibody response to PPRV by species in Tibet, China, 2007*

Species	Region	County	No. serum samples	No. (%) PPRV seropositive
Goat	Ngari	Rutog	198	121 (61.1)
		Gerze	126	56 (44.4)
		Ge'gyai	283	86 (30.4)
		Zada	61	0
		Gar	50	0
		Bulang	45	0
	Others		8	0
Sheep	Ngari	Rutog	11	1 (9.1)
		Gerze	5	3 (60.0)
		Ge'gyai	31	4 (12.9)
		Zada	3	0
		Gar	0	–
		Bulang	23	0
	Others		692	0
Total			1,536	271 (17.6)

*PPRV, peste des petits ruminants virus.

23), indicating high viral loads. However, no animal from Zada County had a Ct <30 (Figure 1).

The study confirmed 11 outbreaks in 4 counties in the southwest Ngari region in Tibet. Nine of the 11 occurred in southern Rutog County, northern Ge'gyai County, and Gerze County, and samples from these 9 were seropositive for PPRV (Figure 1, panel A). By using qRT-PCR and RT-PCR, we found that samples from 4 of these outbreaks were also seropositive for PPRV virus RNA. This finding, confirmed by sequencing, indicates that the main portal of entry for the disease is through southwestern Rutog County. Viral RNA was also detected and confirmed by sequencing in 1 outbreak in southern Ge'gyai County and another outbreak in Zada County (Figure 1).

The nucleic acid sequences obtained from the PCR products were aligned with sequences from PPRV strains available in GenBank. Partial sequencing (448 bp) of the F gene showed that 20 of 21 samples were identical over the portion of the genome that was characterized (GenBank accession no. EU816772). One (GenBank accession no. EU815053) differed from other Ngari sequences by 1 nt. The Ngari sequences showed a level of nucleotide identity with other PPRV strains of 88.8%–98.8%. Strains of PPRV from Tibet were classified as lineage 4 and were closely related to the India/Bsk/Guj/05 strain isolated in India in 2005 (Figure 2, panel A). Partial N-gene sequences (351 bp) from 18 of 19 Ngari samples were identical (GenBank accession no. EU068731), and 1 sequence (GenBank accession no. EU340363) differed by 1 nt. Sequence comparison of the Ngari N gene to the sequences of other PPRV strains showed a nucleotide identity level of 81.6%–97.3%. Strains of PPRV from Tibet were classified as lineage IV and were closely related to the Tajikistan/04 isolate found in Tajikistan in 2004. Different kinds of numbering were used for the phylogenetic

comparisons for the 4 lineages of these 2 genes. The lineages were classified as 1–4 in F gene analyses (*1*). Later research classified the lineages in N gene analyses as I–IV (*2*) (Figure 2, panel B).

Conclusions

In this study, PPRV was found by collecting samples from animals in the field and detecting infection by using competitive ELISA and RT-PCR. Our research provides valuable data on PPRV infection in small ruminants in Tibet. Infection was observed in 4 counties in the Ngari region of southwestern Tibet. Most outbreaks occurred in Rutog and Ge'gyai counties; 1 outbreak was confirmed in Gerze County and another in Zada County. Epidemiologic and serologic evidence suggests that the infection first emerged in Rejiao village in southwestern Rutog from No-
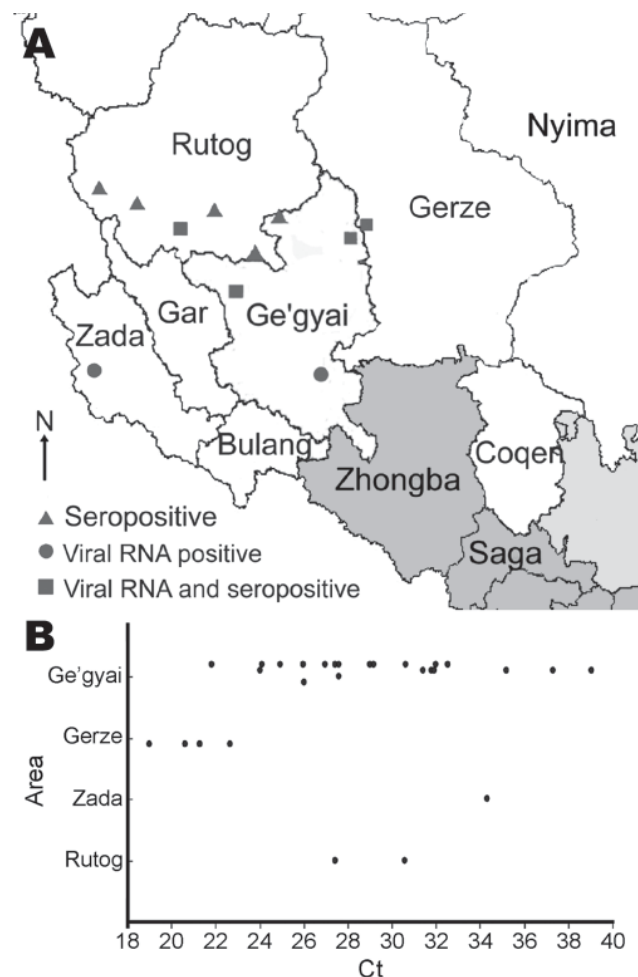

Figure 1. A) Distribution of outbreaks of peste des petits ruminants disease in Tibet, China, 2007. Triangles indicate outbreaks confirmed by ELISA. Circles indicate outbreaks confirmed by reverse transcription–PCR (RT-PCR) and quantitative RT-PCR. Squares indicate outbreaks confirmed by ELISA and molecular methods. B) Cycle threshold (Ct) values (determined by use of q-RT-PCRs on samples) by county.
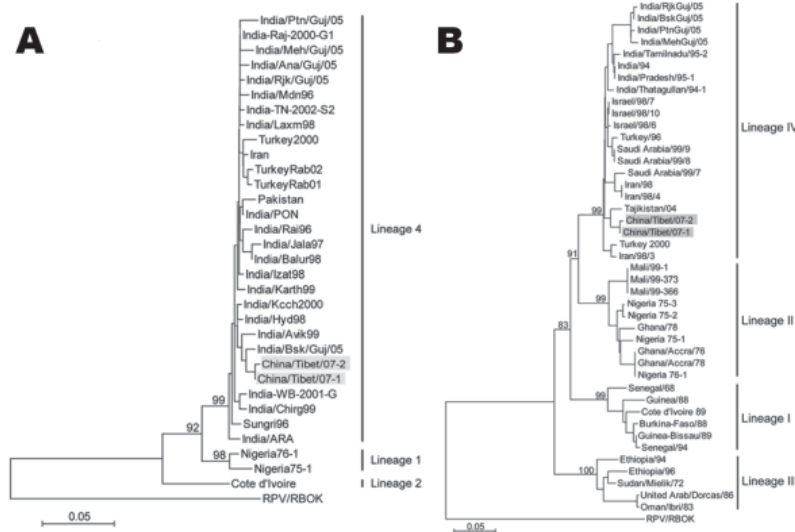
Figure 2. Phylogenetic relationship between peste des petits ruminants virus (PPRV) detected in Tibet, China, in 2007 and other virus isolates. PPRV strains sequenced in this study are highlighted in gray. Other sequences are from GenBank. Phylogenetic analyses were completed with MEGA 3.1 software that used a neighbor-joining algorithm and absolute distances and that followed 1,000 bootstrap replicates. The RBOK vaccine strain of rinderpest virus was included as an outgroup. The tree is based on the partial sequence of the fusion (F) protein gene (A) and the nucleocapsid (N) protein gene (B). Different classifications were used for the phylogenetic comparisons for the West African lineages 1 and 2. Nigeria and related strains have been classified as lineage 1; the Côte d'Ivoire and related strains have been classified as lineage 2 (1). Later research reversed this order in classifying the lineages in N gene analyses (2).

vember 2005 through March 2006. PPR likely existed for several years without being recognized in Tibet because veterinarians, animal health workers, and livestock owners in the area are unfamiliar with its clinical and pathologic features. Also, this disease is frequently confused with other diseases that cause respiratory problems and death in small ruminants (7).

The molecular epidemiologic techniques provided data suggesting cross-border transmission of PPRV infection into Tibet. PPR infection has been recognized in many Asian countries bordering southwestern China, including India (8), Nepal (9), Bangladesh (9), Pakistan (10), and Afghanistan (7). Almost all recent viruses from southwest Asia and the Middle East belong to PPRV lineage 4. The virus that circulated in the Ngari region is of the same lineage and is closely related to an isolate from India (2005) and an isolate from Tajikistan (2004). Close contact between susceptible animals and infected animals in the febrile stage is the main method of transmitting PPR. The terrain of western and southwestern Ngari permits uncontrolled animal movement, and a small ruminant trade exists between Tibet and bordering nations such as India and Nepal. These factors and the history of PPRV in Asia suggest that animals from a neighboring country in southwest Asia are likely sources of this infection in Tibet.

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Mycobacterium bolletii Respiratory Infections

Toïdi Adékambi[1] and Michel Drancourt

Contrary to other species in the *Mycobacterium chelonae-abscessus* complex, we reidentified *M. bolletii* strains isolated from 4 respiratory patients and found these strains to be uniformly resistant to clarithromycin. No mutations previously associated with macrolide resistance in bacteria were detected in either the 23S rDNA or the genes encoding riboproteins L4 and L22.

M*ycobacterium chelonae-abscessus* complex (MCAC) members are opportunistic pathogens in patients with underlying pulmonary disorders (*1–3*). We recently described *M. bolletii* as a new MCAC member (*4*); the pathogen was later isolated from 9% of cystic fibrosis patients (*5*). The initial description of *M. bolletii* suggested that it was highly resistant to antimicrobial drugs, including clarithromycin (*4*). To gain a better appreciation of this resistance, we reidentified MCAC isolates collected in our microbiology laboratory, Timone Hospital, Marseilles, during the past 10 years and performed in vitro susceptibility testing and sequencing of the 23S rDNA, L4, and L22 genes.

Case Reports

Patient 1, a 47-year-old woman with an unremarkable medical history, sought treatment after a 3-week history of hemoptysis; radiographs showed bilateral micronodular infiltrates in the upper lung lobes. Blood tests showed a polymorphonuclear cell count of 9×10^3/mL and biological inflammatory syndrome. Bleeding in the left lobar bronchus was observed during bronchoscopy. Microbiologic results of a bronchial lavage specimen were negative, and microscopic examination found no acid-fast bacilli. An isolate from a subsequent sputum sample was identified later as *M. bolletii*, however. The patient was discharged. No further information is available for this patient.

Patient 2, a 76-year-old man who had received treatment for pulmonary tuberculosis (TB) in 1976, sought treatment in November 1995 for hemoptysis and signs and symptoms of broncho-pulmonary infection. A chest radiograph showed a large cavity in the right upper lobe and infiltrate in the left upper lobe. Two stomach aspirates yielded an isolate identified as *M. abscessus* despite nega-

tive results of a direct microscopic examination; refined identification performed 8 years later in 2003 found both isolates to be *M. bolletii*. The patient received rifampin, clarithromycin, isoniazid, and ciprofloxacin for 16 months. After an initial improvement, the patient continued to have an episodic cough and hemoptysis, and *M. bolletii* was grown from 2 sputum specimens; direct microscopic examination yielded acid-fast bacilli. In November 1998, the patient still had symptoms. In 2003, he was admitted to an intensive care unit for acute respiratory distress syndrome. A broncho-alveolar lavage specimen yielded a mixed culture of multidrug-resistant *M. bolletii* and *Klebsiella pneumoniae* despite negative results of direct examination. The patient eventually died of *K. pneumoniae* septicemia within days of his admission to the ICU.

Patient 3, a 77-year-old man who smoked 3 packs of cigarettes per month and who had a history of pulmonary TB, was admitted to Timone Hospital, Marseilles, in October 2000 with a diagnosis of bronchitis. Corticoid therapy was prescribed. In March 2001, the patient was admitted for respiratory insufficiency. A chest radiograph showed diffuse bullous emphysema in both lungs. Microscopic examination of 1 sputum specimen and 1 bronchial aspirate yielded the presence of acid-fast bacilli that were later identified as *M. bolletii*. The patient left the hospital without treatment, and no further information on his condition is available.

Patient 4, a 90-year-old woman, sought treatment with a temperature of 38°C, hemoptysis, and bilateral micronodular infiltrates of the upper lung lobes. She had a history of childhood pulmonary TB. Sputum specimens yielded mycobacteria that were identified later as *M. bolletii* despite negative results of direct examination. The patient remained febrile after 1 month of treatment with intravenous imipenem and amikacin, her pulmonary condition worsened, and a sputum smear showed numerous acid-fast bacilli. The treatment regimen was changed to a combination of ciprofloxacin, clarithromycin, and ethambutol, but the patient died 2 weeks after treatment began.

The Study

Thirty-one isolates, previously identified as *M. abscessus* (n = 20) and *M. chelonae* (n = 11) by 16S rDNA sequencing from January 1996 through June 2007, were reidentified by partial *rpoB* gene sequencing as described (*6*). The MICs of 22 antimicrobial drugs were determined by E-test (AB Biodisk, Solna, Sweden) after the culture was incubated for 3 days at 30°C. Data were interpreted by using the broth microdilution criteria (*7*). A 1,500-bp fragment of the 23S rDNA (*8*), a 635-bp fragment of the L4 ribosomal protein (forward primer L4Absc7F: 5′-

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ATCGCAGTCAAGGCTCCGG-3′, reverse primer L4Absc642R: 5′-TCAGGCCGACACCTCCTC-3′), and a 471-bp fragment of the L22 ribosomal protein (forward primer L22Absc1F: 5′-ATGACCACTACTACCGAAT-3′, reverse primer L22Absc471R: 5′-CTAGCTGGTGCCTCCCTT-3′) were PCR amplified and sequenced from clarithromycin-resistant isolates. The study was approved by local Ethics Committee, Marseilles Medical School.

Twelve of 31 isolates were identified by *rpo*B sequencing as *M. abscessus*, 11 as *M. chelonae*, 4 as *M. massiliense,* and 4 as *M. bolletii. M. abscessus* and *M. bolletii* isolates showed no intraspecific *rpoB* sequence variation. In contrast, 0.7% sequence divergence was observed in *M. chelonae* (6 sequevars) and *M. massiliense* (3 sequevars) isolates (Figure). The 4 *M. bolletii* isolates were unique among these 31 MCAC isolates in that they were multidrug resistant (Table). The isolates exhibited clarithromycin MICs >256 μg/mL, whereas the other MCAC isolates had clarithromycin MICs ≤2 μg/mL. The E-test is not a validated method for MIC determination in rapidly growing mycobacteria, yet the results we obtained were similar to those previously reported for the reference broth microdilution method (*9*). In the *M. bolletii* isolates, we found no substitutions, deletions, or insertions in domain V (A2058, A2059, C2611 position, *Escherichia coli* numbering) of the 23S rDNA or in the L4 and L22 ribosomal protein genes.

Conclusions

M. bolletii is an emerging pathogen responsible for respiratory tract infections in patients with underlying com-
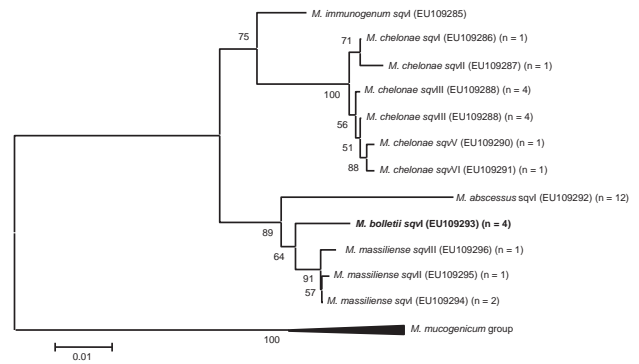

Figure. Phylogenetic tree of different sequevars of *Mycobacterium abscessus-chelonnae* group isolates determined by *rpo*B-723 bp sequencing. Isolate identified as *M. bolletii* is in **boldface**. Scale bar represents 1% sequence divergence.

promised respiratory function. In our study, *M. bolletii* was responsible for pulmonary infection in 3 of 4 patients (patients 2–4) (*10*). *M. bolletii* was repeatedly isolated from different samples from those 3 patients over a period of several weeks. A coexisting broncho-pulmonary disease was found in 3 of the 4 patients, and clinical features and radiograph patterns that suggested nontuberculous mycobacteria infection were observed in all 4 patients (*10*). Infected patients were >75 years of age. Three of 4 patients had hemoptysis and lung infiltrates, *M. bolletii* was the sole organism isolated from respiratory tract specimens. In this study, 16S rDNA sequencing misidentified 25% of

Table. Antimicrobial drug susceptibility test results (MICs) for *Mycobacterium bolletii* isolates*				
Antimicrobial agent	Patient 1	Patient 2	Patient 3	Patient 4
Penicillin	>32	>32	>32	>32
Amoxicillin	>256	>256	>256	>256
Amoxicillin clavulanate (disc 20 μg + 10 μg)	>4	>4	>4	>4
Cefoxitin	>256	>256	>256	>256
Ceftriaxone	>256	>256	>256	>256
Cefotaxime	>256	>256	>256	>256
Imipenen	>32	>32	>32	>32
Doxycycline	>32	>32	>32	>32
Minocycline	>256	>256	>256	>256
Clarithromycin	>256	>256	>256	>256
Erythromycin	>256	>256	>256	>256
Azithromycin	>256	>256	>256	>256
Amikacin	32	48	32	48
Tobramycin	>32	>32	>32	>32
Ciprofloxacin	>32	>32	>32	>32
Ofloxacin	>32	>32	>32	>32
Sparfloxacin	>32	>32	>32	>32
Rifampicin	>32	>32	>32	>32
Metronidazole	>256	>256	>256	>256
Teicoplanine	>256	>256	>256	>256
Vancomycin	>256	>256	>256	>256
Trimethoprim-sulfamethoxazole	>32	>32	>32	>32

*Values given in μg/mL.

31 MCAC isolates that were eventually identified as *M. massiliense* and *M. bolletii*. This statistic agrees with observations made during the recent description of *M. bolletii* infection after mesotherapy (*11*).

Clarithromycin was administrated to 2 of the 4 patients, both of whom died within several weeks after treatment began. The 4 *M. bolletii* isolates were highly resistant to clarithromycin, yet they did not harbor the 23S rDNA mutations that have been previously found in clarithromycin-resistant *M. abscessus* strains (*12*). Additionally, no mutations in riboproteins L4 and L22, which are associated with macrolide resistance in *Streptococcus pneumoniae* (*13*), were detected. RNA methylase genes *erm*[38], *erm*[39], and *erm*[40], which confer inducible macrolide resistance in *M. fortuitum*, *M. smegmatis*, *M. mageritense*, and *M. wolinskyi,* are absent in the MCAC (*14*). Further investigations are therefore needed to clarify the mechanism of clarithromycin resistance in *M. bolletii*.

Clarithromycin has been recommended as the first-line antimicrobial drug for treating rapidly growing mycobacteria infections in patients with compromised respiratory function (*1,10*). Patient deaths (*3*) have been linked to clarithromycin resistance, with a risk of secondary clarithromycin resistance during monotherapy estimated to be <10% (*12*). Recent studies showed that 21%–36% of MCAC isolates were resistant to clarithromycin (*15*). The recommendation for treating *M. abscessus* infection with clarithromycin was made before discovering *M. bolletii*, a multidrug-resistant species that mimics *M. abscessus*. Our report illustrates that accurate species identification and in vitro clarithromycin susceptibility testing should be recommended for MCAC isolates of clinical interest. GenBank accession nos. were as follows: for 23S rDNA sequences, *M. bolletii* CIP 108541^T (EU109306), *M. massiliense* CIP 108297^T (EU109307), *M. abscessus* CIP 104536^T (EU109308), and *M. chelonae* CIP 104535^T (EU109309); for L4 sequences, *M. bolletii* CIP 108541^T *M. massiliense* (EU779956), CIP 108297^T (EU779957), *M. abscessus* CIP 104536^T (EU779957), and *M. chelonae* CIP 104535^T (EU779958); and for L22 sequences *M. bolletii* CIP 108541^T (EU779952), *M. massiliense* CIP 108297^T (EU779953), *M. abscessus* CIP 104536^T (EU779954), and *M. chelonae* CIP 104535^T (could not be amplified with the designed primers).

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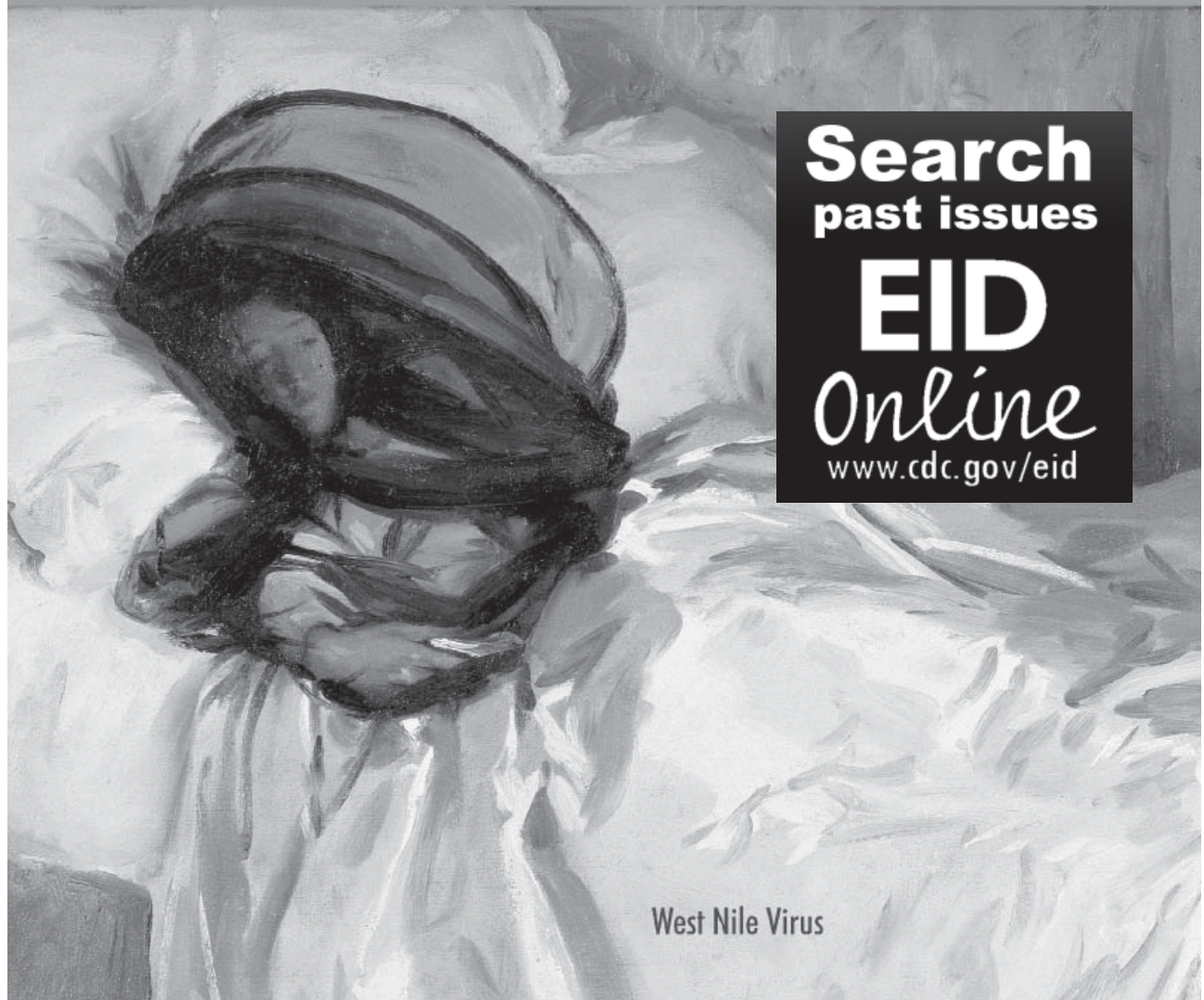
Tahyna Virus and Human Infection, China

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In 2006, Tahyna virus was isolated from *Culex* spp. mosquitoes collected in Xinjiang, People's Republic of China. In 2007, to determine whether this virus was infecting humans, we tested serum from febrile patients. We found immunoglobulin (Ig) M and IgG against the virus, which suggests human infection in this region.

Tahyna virus (TAHV) has been reported to cause human illness throughout much of Europe and Asia, including countries adjacent to the border of northwestern China (*1–4*). TAHV (family *Bunyaviridae,* genus *Orthobunyavirus*, California serogroup) was first isolated in the former Czechoslovakia in 1958 from a pool of *Aedes caspius* mosquitoes (*5*). TAHV is widely distributed in central Europe, as shown by the following: numerous virus isolates have been obtained from mosquitoes, TAHV-specific antibodies have been detected in several nonhuman mammals and several avian species, and TAHV antibodies are highly prevalent in humans in some localities (*6*). Human illness from infection with TAHV has been reported as manifesting undifferentiated fever and influenza-like symptoms; the infection may also cause pneumonia and pleurisy, acute arthritis, pharyngitis, and, occasionally, central nervous system involvement (*6,7*).

The presence of TAHV in China was postulated after antibodies were detected in serum specimens collected from healthy adults in Xinjiang Province in 1985 (*8*),

although a subsequent study of the cause of encephalitis cases across China during 1988–1990 failed to detect seroconversion to any California group viruses (*9*). Here we report isolation of TAHV in China, along with evidence of TAHV infections in humans. Human infection can be inferred from positive serologic results of samples taken from persons who visited an outpatient clinic in the same region from which the TAHV isolate was derived.

The Study

Mosquitoes were collected in several counties in the Kashi area, Xinjiang Uygur Autonomous Region, China (Figure 1, panel A), from July through August 2006. Mosquitoes were collected in light traps, identified, and sepa-

Figure 1. A) Map of China showing location of Xinjiang Uygur Autonomous Region. B) Map of Xinjiang Uygur Autonomous Region showing Kashi region (star), where Tahyna virus XJ0625 was isolated from a pool of *Culex* spp. mosquitoes.

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rated by genus/species/collection site into pools of up to 100 specimens. Species collected included *Culex annulirostris, Cx. quinquefasciatus,* and *Cx. pipiens.* Unfortunately, several specimens could not be identified below genus. A total of 9,865 mosquitoes were tested in 100 pools that contained both unfed and blood-fed specimens.

Specimens were triturated in minimal essential medium, clarified by centrifugation, and added to confluent sheets of baby hamster kidney (BHK)–21 and Vero cells in 6-well plates, which were incubated at 37°C (*10*). A single pool containing 100 specimens of unidentified *Culex* spp. mosquitoes collected from Tierimu town, Jiashi county, Kashi (39°52′87″N, 76°71′51″E) (Figure 1, panel B), yielded a virus isolate designated XJ0625. Because the pool contained unfed and blood-fed mosquitoes, the virus isolate may have come from either an infected mosquito or from infected animal blood in the mosquito's gut. At 24 h after the mixture was added to the 6-well plates, this isolate produced giant cells and syncytia in both BHK and Vero cells. The cell monolayer was destroyed rapidly, within 24 h of the first cytopathic effects. After intracranial inoculation with XJ0625, suckling mice showed signs of tremor and stiff neck at 24 h and died within 48 h.

RNA was extracted from an XJ0625 RNA lysate and subjected to reverse transcription–PCR (RT-PCR) amplification by using genus primer sets designed for the detection of flavivirus, alphavirus, and bunyavirus RNA (*11–13*). The sequence amplified by the bunyavirus genus primer had a high homology with TAHV. Subsequently, microplate plaque-reduction neutralization tests (*14*) were performed with BHK-21 cells and immune ascites fluid with immunity to prototype TAHV (Bardos 92; provided by the Centers for Disease Control and Prevention (CDC), Fort Collins, CO, USA) to validate the molecular identification. XJ0625-associated cytopathic effects were completely inhibited at ascites fluid dilutions up to 1:3,200.

The nucleotide sequence of the small (S) (EU622820) and medium (M) (EU622819) segments of XJ0625 were sequenced by using the primers SF (5′-AGTAGTGTACCCCACTTGAAT AC-3′), SR (5′-CAA ATGGATTTGATCCTGATGC-3′), M1F (5′-CACAAGT TCCAAGATGATGTT-3′), M 1R (5′-CTGTGCCTTCTGC TTGGACTA-3′), M2F (5′-GTCCAAGCAGAAGGCAC AGAT-3′), M2R (5′-GTGGTCACTGTACATTCTCC TGAA-3′), M3F (5′-CACACTTCTGTTTAGCAGATA CC-3′), M 3R (5′-CTCTAGTCTATAGCTTGCTG GTG TT-3′), M4F (5′-GCACCAATCTGAACGCAATAACAC-3′), and M4R (5′-AGTAG TGTGCTACCAAGTATA-3′). By using Clustal X version 1.8 (www.clustal.org), sequences were aligned with those of viruses belonging to the California virus group. The phylogenic status of XJ0625 isolate was assessed by using MEGA version 3.1 software

(www.megasoftware.net), and phylogenetic trees were constructed by using the neighbor-joining algorithm with 1,000 bootstrap replicates.

Phylogenetic analyses of the nucleotide sequences of the S (Figure 2, panel A) and M (Figure 2, panel B) segments generated highly comparable topologies, which indicates that XJ0625 has a high level of sequence homology with TAHV. To provide independent confirmation, we sent the XJ0625 viral RNA to CDC, Fort Collins, Colorado, USA, for further characterization. RNA was subjected to RT-PCR amplification by using multiple primer sets designed for the detection of orthobunyavirus S segment RNA (*11*). Nucleotide sequencing of amplified DNA fragments that, in combination, span the entirety of the TAHV S segment was used to positively identify XJ0625 viral RNA as TAHV RNA (data not shown) (*15*).
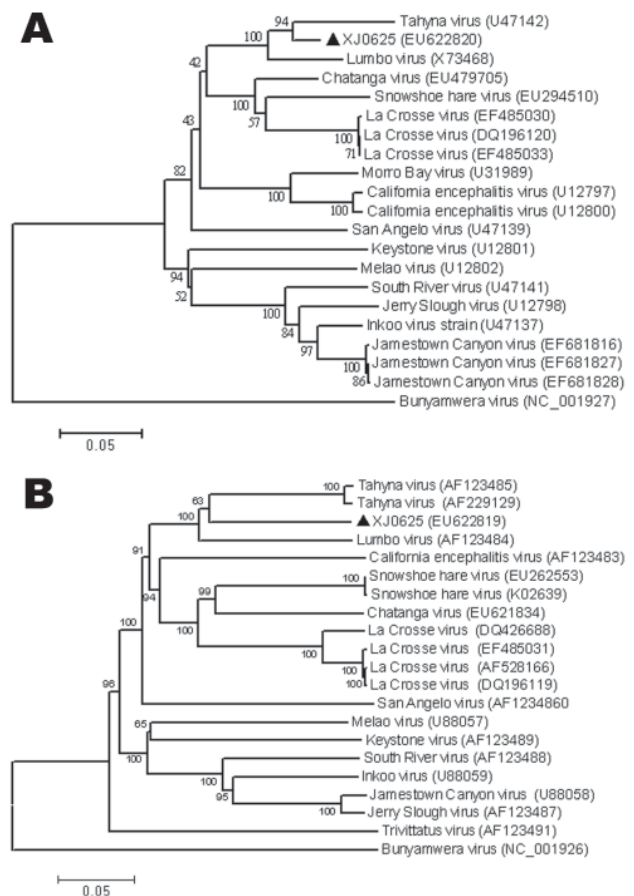

Figure 2. Phylogenetic analysis of Tahyna virus (TAHV) XJ0625 from China based on the complete nucleotide sequence of the small segment (A) and the medium segment (B). Distances and groupings were determined by the p-distance algorithm and neighbor-joining method with MEGA version 3.1 software (www.megasoftware.net). Bootstrap values are indicated and correspond to 1,000 replications. The tree was rooted by using Bunyamwera virus as the outgroup virus. Scale bars indicate a genetic distance of 0.05-nt substitutions per position.

To determine whether persons in the region were becoming infected with TAHV, we collected serum samples from 323 persons who visited an outpatient clinic in Jiashi County and its adjacent counties in Kashi from August 18 through September 20, 2007. Specimens were collected within 1–3 days of onset of clinical signs and symptoms, which consisted of fever (37°C–39°C) in all patients and headache with no other specific symptoms in a few. The serum specimens were screened by indirect immunofluorescence assay by using XJ0625-infected BHK-21 cells. Infected and uninfected cell suspensions were applied to Teflon-coated, 10-well slides, air dried, and fixed. The serum specimens were applied to the spot slides at dilutions of 1:40 for IgM detection and 1:80 for IgG detection. After incubation and washing, the spot slides were treated with fluorescein-conjugated antihuman IgM or IgG, dried, and examined by fluorescent microscope. Of the 323 samples, 42 (13.0%) were IgG positive and 17 (5.3%) of 323 were positive for both IgM and IgG against XJ0625 (Table).

To determine whether the initial illness of the patients was associated with TAHV infection, in December 2007 (95–127 days after collection of the first sample) we obtained a second serum sample from 10 of the IgM-positive patients. These paired serum specimens were tested by serum dilution neutralization test with XJ0625 virus on BHK-21 cells. Serial 2-fold dilutions of serum were added to equal volumes of culture medium containing XJ0625 virus (50% tissue culture infective dose on a 96-well microtiter plate) and incubated at 37°C for 1 h; 100 μL of each virus plus diluted serum was added to 4 wells of a 96-well tissue culture plate containing confluent monolayer of BHK-21

cells. After the cultures were incubated for 48 h at 37°C, we calculated the antibody titer as the highest dilution at which cytopathic effects were completely inhibited in the well. Most of the samples contained neutralizing antibody at a titer of 40 or 80 (Table), though no samples produced a 4-fold titer change that would suggest that TAHV infection caused the initial illness.

Conclusions

We isolated TAHV from mosquitoes in China and detected IgG consistent with TAHV antibodies in 13.0% of the samples, which suggests that human infection with TAHV is common in the area. We recognize that further testing is necessary to determine whether this serologic response was due to TAHV or to another California serogroup orthobunyavirus.

These results indicate that in northwestern China, TAHV should be investigated as a possible etiologic agent in cases of febrile illness with pulmonary involvement and in cases with central nervous system involvement not attributable to other causes. Research should be conducted to identify the primary mosquito vectors and vertebrate amplifier hosts in this region. Additionally, TAHV should be investigated as a cause of illness in other areas of China.

Table. Results of serologic testing of human serum samples for the presence of antibody against TAHV XJ0625, 2007*

Case no.	Sex/age, y	Dates of serum sample collections	Days between serum sample collections	IgM (IFA)	IgG (IFA)	Neutralizing antibody titer
1	M/20	Sep 5	103	+	+	80
		Dec 17		NT	NT	40
2	M/72	Sep 5	103	+	+	80
		Dec 17		NT	NT	80
3	M/7	Sep 5	104	+	+	40
		Dec 18		NT	NT	10
4	M/25	Sep 6	103	+	+	80
		Dec 18		NT	NT	80
5	F/27	Sep 6	103	+	+	40
		Dec 18		NT	NT	40
6	M/35	Aug 18	122	+	+	40
		Dec 18		NT	NT	40
7	M/7	Aug 24	116	+	+	40
		Dec 18		NT	NT	80
8	F/43	Aug 27	113	+	+	40
		Dec 18		NT	NT	40
9	F/60	Aug 31	111	+	+	80
		Dec 20		NT	NT	80
10	F/42	Sep 16	95	+	+	80
		Dec 20		NT	NT	80

*TAHV, Tahyna virus; Ig, immunoglobulin; IFA, immunofluorescence assay; NT, not tested.

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Novel Human Parechovirus from Brazil

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Human parechoviruses (HPeVs) were detected by reverse transcription–PCR in 16.1% of 335 stool samples from children <6 years of age with enteritis in Salvador, Brazil. Whole genome sequencing of 1 sample showed a novel HPeV that has been designated as HPeV8.

The human parechovirus (HPeV) species are small, nonenveloped RNA viruses that belong to the highly diversified family *Picornaviridae* (*1*). HPeV types 1 and 2 had been known as echoviruses 22 and 23 within the genus *Enterovirus* but were recognized in the early 1990s as an independent genus (*2*). Recognition of clinical relevance is increasing after 4 novel types were more recently described (*3–7*).

Seroprevalence studies from different countries indicate that almost the entire human adult population is infected. Predominantly in infants, HPeVs can cause a variety of clinical symptoms, including diarrhea, and respiratory infection (*8,9*). Recent data point toward substantial involvement in severe conditions, such as meningitis and infant sepsis, for which HPeV may constitute the second most frequent causative virus after enterovirus in young children (*10*). Different HPeV types may cause different clinical diseases (*10,11*). Unconnected diseases might be caused by yet unrecognized HPeVs.

The Study

To identify possibly unrecognized HPeVs, we systematically searched for HPeVs in patients in Brazil with enteritis. We used stool samples for the study because the related enteroviruses are preferentially transmitted through feces. Because reverse transcription–PCR (RT-PCR) for the ge-

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nus *Enterovirus* cannot detect HPeVs (*9,10*), a broad-range real-time RT-PCR assay was developed that can detect all known parechovirus types. The assay also can detect new HPeV types (*9*).

The study cohort comprised 335 stool samples from Brazilian infants and children ≤6 years of age with acute diarrhea, defined as >3 watery stools in the previous 24 hours and lasting no longer than 13 days. From February 2006 through August 2007, children were seen as outpatients or were hospitalized at the University Hospital in Salvador de Bahia, Brazil, because of severe dehydration. All analyses were performed by using an ABI 7500 real-time RT-PCR (Applied Biosystems, Foster City, CA, USA) and an ABI 3100 automated sequencing platform (Applied Biosystems) at the local Infectious Disease Research Laboratory. Informed consent was obtained from the mothers of all enrolled patients. The study was approved by the institutional ethics committee.

We performed RNA extraction and real-time RT-PCR as described (*9*). A total of 16.1% of samples tested positive for HPeV. Many samples yielded low HPeV RNA concentrations indicated by threshold cycles later than 32 in real-time RT-PCR.

The viral protein (VP) 1 capsid protein gene has been established for molecular typing of HPeV (*3*). Consensus primers for amplification and sequencing of the VP1 gene were developed. Primer sequences were VP1 forward 5′-CCATARTGYTTRTARAARCCYCT-3′ and VP1 reverse 5′-CARAAYTCDTGGGGYTCMCARATGG-3′. VP1 RT-PCR was successful in only 11 of 54 HPeV-positive samples, consistent with low RNA concentrations in most samples. Ten of the 11 sequenced samples were of known and well-characterized types (type 1, 7 samples; type 5, 2 samples; and type 6, 1 sample). These samples were not further analyzed. One sample showed a VP1 sequence that clustered with none of the known HPeV types in phylogenetic analysis (Figure 1). To determine whether this virus represented a new type, its complete genome except the first 27 nucleotides of the 5′ terminus was amplified by overlapping PCR fragments, and the full nucleotide sequence was determined as described previously (*9*) (GenBank accession no. EU716175).

The best matching sequence was HPeV type 4 with 76.3% aa identity (Table). Lowest aa identity was 69.3% between the new sequence and type 5. Genetic identity to Ljungan virus, a rodent parechovirus, was only 44.5%, comparable to all previously described HPeVs. Phylogenetic segregation from all known HPeVs was obvious not only in VP1 but also along the structural proteins VP0 and VP3 (aa identities with established HPeV types ranges 71.2%–80.2% in VP0 and 74.1%–80.0% in VP3) (Figure 1).

Nonstructural family *Picornaviridae* genes are highly recombined (*1,3,12,13*), resulting in a mosaic structure that
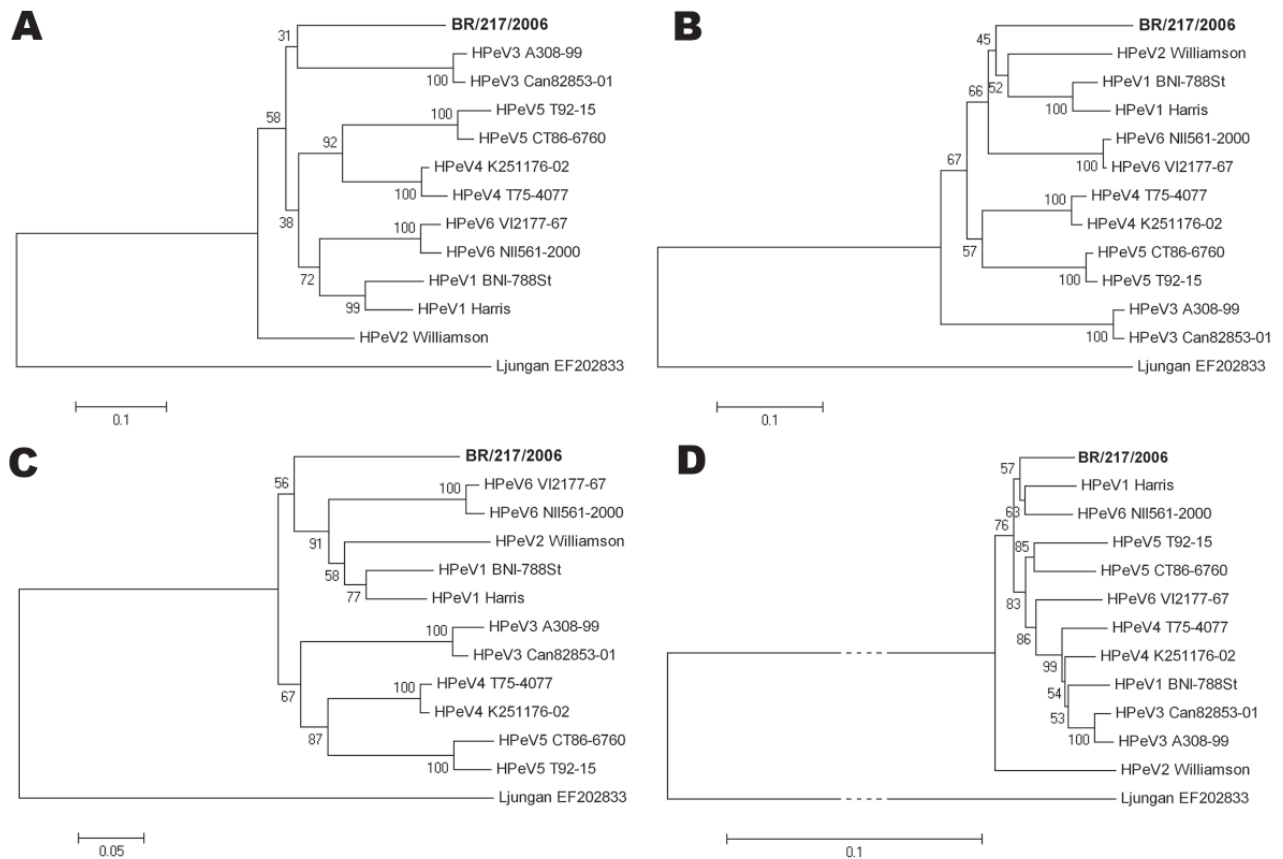
Figure 1. Evolutionary relationships between known parechoviruses and the new human parechovirus from this study (shown in **boldface**). Phylogenetic analyses of A) complete viral protein (VP) 1, B) VP0, and C) VP3, and D) the whole nonstructural region (comprising regions P2 and P3), were constructed using the Jones-Taylor-Thornton matrix-based substitution model on an amino acid–driven alignment (pairwise deletion option). The evolutionary histories were inferred using the neighbor-joining method and are in the units of the number of amino acid substitutions per site. Relevant bootstrap values from 500 replicate trees are shown next to the branches. The scale bars show the evolutionary distance from each root. Analysis was conducted in MEGA software version 4 (www.megasoftware.net). HPeV reference strains are named in full detail; their GenBank accession numbers are not further indicated. Contemporary HPeV1 strain BNI-788st accession number was EF051629 and HPeV6 strain BNI-67 accession number was EU022171. The tree was rooted against Ljungan virus, a rodent parechovirus (GenBank accession no. EF202833). In the P2/P3 tree, branches to the Ljungan virus node have been truncated for space reasons, as indicated by dotted lines.

limits their utility in phylogenetic analysis. This characteristic also was the case for the whole nonstructural region (comprising regions P2 and P3) of the novel virus. It was not clearly segregated from that of all other HPeV prototype strains, showing closest overall relationship with HPeV type 1 strain Harris and the HPeV6 prototype strain NII561-2000 (Figure 1). To identify possible recombination in the P2/3 region of the novel virus, we conducted Sim Plot analysis (http://sray.med.som.jhmi.edu/SCRoftware/simplot) (Figure 2). We found no clear similarity with any of the established HPeV prototype strains. In contrast to other HPeV prototype strains, BootScan analysis showed no evidence for recombination with other prototype strains in the nonstructural gene region. However, this analysis could also not exclude recombination with any other HPeV because of the small number of HPeV full genome sequences currently available.

The new HPeV lacked a typical RGD (Arg-Gly-Asp) aa motif in the VP1 C terminus. This motif has proven important for HPeV type 1 infectivity, presumably because of interaction with cellular receptors (*14*). Such a motif is present in all known HPeV strains except type 3, and some researchers have suggested that the latter may use a different receptor for cell entry (*3,13*).

Conclusions

Most HPeV types have been identified only recently. The associated spectrum of diseases is not fully understood and probably has been underestimated. Recent data indicate that HPeVs may cause severe clinical conditions, such as infant sepsis and meningitis, in addition to acute diarrhea (*10*). Prevalence in young children with diarrhea was ≥16% in previous studies; more important, ≤8% of meningitis

Table. VP1 amino acid identity between sequences of HPeV, Brazil*

PeV type and strain	1	2	3	4	5	6	7	8	9	10	11	12	13
HPeV1													
1. Harris													
2. BNI-788St	89.6												
HPeV2													
3. Williamson	78.7	80.0											
HPeV3													
4. A308-99	71.7	71.7	71.7										
5. Can82853-01	72.6	72.1	71.7	97.3									
HPeV4													
6. T75-4077	78.4	74.0	74.8	70.4	71.2								
7. K251176-02	77.9	77.1	76.5	70.4	70.8	96.6							
HPeV5													
8. CT86-6760	74.9	74.0	73.0	66.4	66.8	77.6	80.2						
9. T92-15	74.9	71.4	70.9	65.0	65.5	76.7	77.2	94.8					
HPeV6													
10. NII561-2000	81.4	78.8	73.5	74.8	75.2	73.2	73.2	71.4	71.9				
11. VI2177-67	81.4	77.9	73.5	73.5	73.9	73.2	73.2	72.7	73.2	95.7			
Novel type													
12. BR/217/2006	74.6	75.9	73.7	72.0	72.4	74.1	76.3	72.4	69.3	72.8	72.8		
Rodent PeV													
13. Ljungan	43.5	43.5	48.0	44.4	44.0	42.9	44.6	42.9	42.4	43.1	43.5	44.5	

*HPeV, human parechovirus; PeV, parechovirus; VP1, viral protein 1. Percentage of amino acid identity per site from analysis between sequences is shown. All results (%) are based on the pairwise analysis of 13 sequences (pairwise deletion option). Analyses were conducted in MEGA software (www.megasoftware.net). There were 297 positions in the final dataset.

cases showed evidence of HPeV (*9–11*). The molecular ecology of HPeV seems especially relevant in view of their diversified and strain-dependent pathogenesis (*10,11*).

This report on HPeVs from Brazil confirms their global distribution. The level of diversification between the novel parechovirus and established HPeV types is clearly higher than the 20% aa distance in the VP1 protein, which resembles the distance between serotypes of enteroviruses

(*1,3*) and exceeds the definition threshold of HPeV types (*3*). During revision of this report, the virus received the designation HPeV8 by the ICTV Picornavirus Study Group (www.picornastudygroup.com/types/parechovirus/hpev. htm). Like HPeV3, HPeV8 lacks the RGD motif; some researchers have suggested that HPeV3 may use a different receptor than other HPeV types for cell entry (*3,13*). Of all HPeV types, type 3 has been most strongly asso-
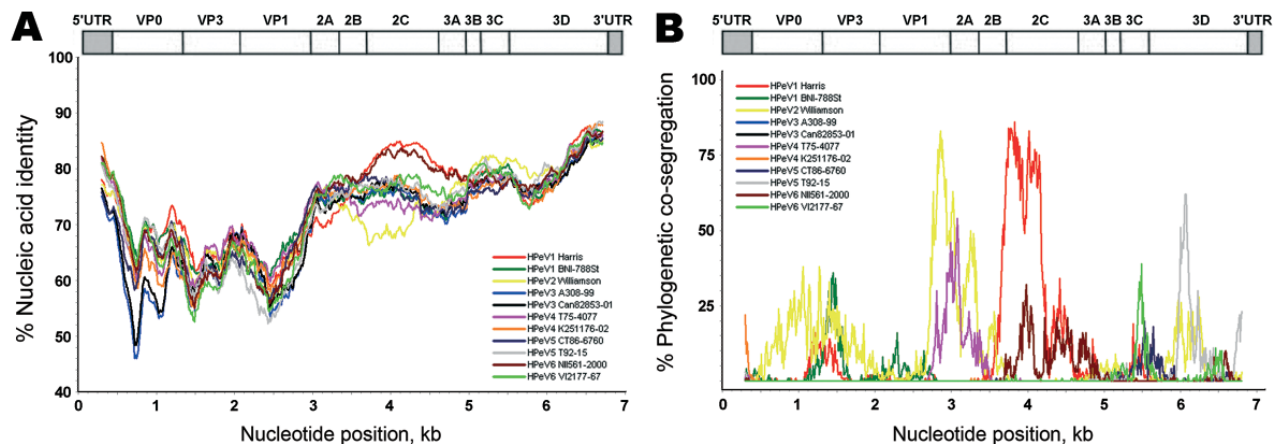

Figure 2. Nucleic acid identity of the new parechovirus with known parechoviruses. The near full-length genome of the new parechovirus BR/217/2006 was analyzed with SimPlot software (http://sray.med.som.jhmi.edu/SCRoftware/simplot) using a 600-bp sliding window and a step size of 10. Because of partially incomplete 5′ untranslated region (UTR) GenBank reference sequences, an approximate 400 nucleotides had to be cut from the 5′ end of all genomes. HPeV, human parechovirus. A) Nucleic acid identity, per analysis window, for strain BR/217/2006 with prototype strains. Nucleotide positions on x-axis show the center of the window. B) BootScan analysis using the same window settings. A bootstrapped phylogenetic analysis was conducted per window along the alignment. Graphs represent the percentage (bootstrap values) at which each strain cosegregates phylogenetically in the analysis window with strain BR/217/2006. Prototype strains used for comparison are shown in the inserts in each panel. A schematic representation of the parechovirus genome is given on top.

ciated with severe neurologic and systemic clinical conditions (*10,11,15*). The lack of an RGD motif might implicate a different cell or tissue tropism for HPeV8 as well. The search for unknown HPeVs should be extended to other clinical conditions thus far not associated with HPeV.

Acknowledgments

Dr Drexler is a physician and clinical virologist affiliated with the University of Bahia, the Bernhard Nocht Institute, and the University of Bonn. His research interests are the implementation of methods for affordable viral load monitoring and the characterization of novel human and zoonotic viruses.

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Reemergence of Human and Animal Brucellosis, Bulgaria

**Gianluca Russo,[1] Paolo Pasquali,[1]
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Stanislav Ralchev, Vincenzo Vullo,
Giovanni Rezza, and Todor Kantardjiev**

Bulgaria had been free from brucellosis since 1958, but during 2005–2007, a reemergence of human and animal disease was recorded. The reemergence of this zoonosis in the country highlights the importance of maintaining an active surveillance system for infectious diseases that will require full cooperation between public health and veterinary authorities.

According to the World Health Organization (*1*), brucellosis is one of the most common zoonoses worldwide and is considered a reemerging infectious disease in many areas of the world. An estimated 500,000 new human cases occur annually worldwide (*2*). In Europe, 1,033 human brucellosis cases were reported in 2006 (*3*); data from a passive surveillance system were based on clinical findings, supported by epidemiologic criteria, and confirmed by serologic tests. Here we report the results of a survey performed in Bulgaria during 2005–2007, which has been considered free from *Brucellosis melitensis* and *B. abortus* disease since 1958 (*4*).

In Bulgaria, until 1998 serologic screening was mandatory for all cattle, sheep, and goats >12 months of age. Afterward, based on risk assessment, animal surveillance activities covered 100% of heads reared in municipalities along the borders with countries endemic for brucellosis such as Turkey, Greece, and the former Yugoslav Republic of Macedonia; 50% of the animals reared in other municipalities of the regions bordering the aforementioned countries; and 25% of animals reared in the inner Bulgarian regions. Currently, an active surveillance system is in place for dairy factory employees and persons considered at risk after outbreaks in ruminants.

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The Study

During 2005–2007 (Figure 1), a total of 105 human cases of brucellosis were diagnosed among 2,054 persons

Figure 1. Geographic distribution of human and animal brucellosis in Bulgaria during A) 2005, B) 2006, and C) 2007.

[1]These authors equally contributed to this article.

who were tested on the basis of clinical suspicion or risky exposure. A human case of brucellosis was considered confirmed if results of serologic tests, such as ELISA or complement fixation test, were positive, in accordance with the World Health Organization case definition (*5*). Bacteria isolation and characterization had not been performed routinely.

The alert started in 2005 (Figure 1, panel A), when a case of brucellosis occurred in a Bulgarian migrant animal keeper working in Greece. Active surveillance of persons at risk was implemented, enabling detection of a total of 34 human cases of brucellosis. All cases were classified as imported cases; therefore, no supplemental active surveillance on animals was implemented. Additionally, during routine screening for at-risk workers, 3 other persons employed in a dairy factory were found to be seropositive. Due to the lack of traceability of the raw material used in the factory, it was not possible to trace the origin of the infection. At that time, there was no evidence of animal cases of brucellosis.

During 2006 (Figure 1, panel B), 10 cases of human brucellosis were reported from different regions of the country. According to anamnestic information, these case-patients had different sources of infection: 3 of the 10 were considered imported infections; 1 case-patient was diagnosed during hospitalization in Sicily (Italy), where the patient reported having eaten ricotta cheese, and 2 occurred in Bulgarian migrant animal keepers working in Greece. Concerning the origin of infection, epidemiologic data suggest that 5 of the 10 cases were related to occupational risk and the remaining to consumption of raw milk and milk derivates. Surveillance activities enabled detection of 10 animals (7 small ruminants and 3 cows) with positive serologic results; these animals were then killed and destroyed. During 2007 (Figure 1, panel C), a total of 58 human cases were identified. Of 58 cases, 54 were classified as autochthonous (i.e., acquired by imported animals found to be infected during regular veterinary surveillance). These cases were identified in a Bulgarian region bordering Greece and Turkey (Haskovo region).

Two other cases, which were also classified as autochthonous, were diagnosed in patients who stated they had consumed a risky product (i.e., raw milk handled without adherence to hygienic standards). The remaining 2 cases were classified as imported because they involved Bulgarian migrant animal keepers working in Greece. Active surveillance in place for animals found a total of 625 heads (618 small ruminants, 7 cows) with positive serologic results; all were killed and destroyed. Analogous with what we observed in humans, most of the infected animals were found in the Haskovo region. All animals found to be infected during surveillance activity were bred at the family farm, and their milk and dairy products were prepared and eaten without adherence to proper hygienic standards.

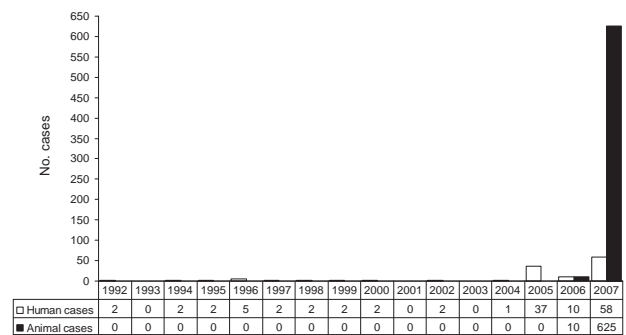|	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
□ Human cases	2	0	2	2	5	2	2	2	2	0	2	0	1	37	10	58
■ Animal cases	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	625

Figure 2. Human and animal cases of brucellosis in Bulgaria, 1992–2007. In Bulgaria, during 1992–2004, a total of 22 human cases and 0 animal cases of brucellosis were recorded; during 2005–2007, a total of 105 human cases and 635 animal cases of brucellosis were recorded.

Conclusions

Our data show that brucellosis is reemerging in Bulgaria (Figure 2). On the basis of information provided in this report, we can make several hypotheses regarding the causes of the resurgence of a previously controlled infection in a transitional, rapidly changing country.

Overall, 105 human cases of human brucellosis were identified over a 3-year period. Of them, 84 cases (80%) were identified in persons at occupational risk. This finding suggests that when brucellosis is introduced into naive territories (i.e., those territories that were considered officially free of brucellosis), the primary source of infection for humans is direct contact with infected animals (i.e., exposure to abortion/delivery products) or domestic consumption of products produced on family farms (milk, raw cheese). However, environmental exposure can also occur, especially in infants and children, who are considered at lower risk for direct contact with potentially infected animals, as recently observed (*6*). This hypothesis appears to be consistent with the context of a naive setting, where preventive measures are not routinely implemented. Continuous health education and other strategies may contribute to reduce the circulation of human brucellosis in endemic areas (*7*).

The reemergence of brucellosis is not limited to Bulgaria but involves several countries in the Balkan region and even in the Caucasian region (P. Pasquali, unpub. data). This trend or reemergence has several explanations. First, due to socioeconomic changes, many countries in these regions are experiencing a dramatic increase of animal trade, animal movement, and occupational migration, which in turn may increase the risk for introduction and spread of infectious diseases, such as brucellosis, from other disease-endemic countries like Greece or Turkey (*2*). Second, the process that has characterized the change of the social and administrative organization since the collapse of the Soviet Union is far from being completed; the public

health systems are still flawed in many countries. Finally, part of the increase may simply be that brucellosis is a complex disease, which has different cycles of expansion and regression.

Before drawing conclusions, we should mention 2 possible limitations of the study. First, samples from patients with positive serologic results were used for bacterial culture for brucellosis only if sample collection was properly timed; no culture positive case is available. Second, we cannot exclude the possibility that part of the increase in cases of brucellosis could be due to improved surveillance; in particular, temporal trends and geographic comparison might be, to some extent, affected by the intensity of screening activities. However, this increased surveillance is unlikely to bias the observed shift from imported to locally acquired cases.

In conclusion, this report shows how a disease such as brucellosis may increase its public health impact, particularly in transitional countries such as Bulgaria. Our findings emphasize the importance of the combination of health education and active surveillance systems for controlling infectious diseases and highlight the need for cooperation between public health officials and veterinary officers. Creating and improving capacity building are necessary to properly address issues that pose public health hazards.

Dr Russo is a physician at Sapienza University in Rome. His research interests are infectious diseases in resource-limited countries in the context of natural disasters.

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Epizootic Hemorrhagic Disease in Cattle, Western Turkey

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Peter Paul Clement Mertens, and Hasan Batmaz

In 2007, an outbreak of epizootic hemorrhagic disease (EHD) occurred in Turkey. On the basis of clinical investigation, 41 cattle were suspected to have EHD. Reverse transcription–PCR and sequence analyses indicated that the virus belonged to EHD virus serotype 6, thus confirming EHD virus infection of cattle in Turkey.

Epizootic hemorrhagic disease virus (EHDV) is a member of the genus *Orbivirus*, family *Reoviridae,* and is closely related to bluetongue viru*s* (BTV). EHD often causes death in white-tailed deer and, less frequently, a bluetongue-like illness in cattle (*1–3*).

Culicoides spp. act as vectors, transmitting EHDV between susceptible ruminant hosts (*2*). The clinical signs of EHD in cattle are fever, anorexia, dysphagia, ulcerative and necrotic lesions of the oral mucosa (Figure 1), hyperemia and edema of the conjunctival mucosae (Figure 2), sore muzzle, hyperemia of the teats and udder, hemorrhage, dehydration, and lameness (*3*). EHDV has been isolated from cattle throughout the world, including Africa, North America, Australia, Japan, and recently Israel (*4–10*). Recent outbreaks of EHDV in Israel during 2006 were attributed to EHDV-7 (*6*); outbreaks in Morocco and Algeria were similar to EHDV-6/EHDV-318. An initial suspicion of EHD, based on observation of clinical signs, can be confirmed by virus isolation and characterization, nucleic acid identification, or serologic testing. ELISA is a specific and sensitive method for detecting EHDV-specific antigens or antibodies and confirming the disease (*2–5,8,11,12*).

The Study

In July 2007, a 7-week outbreak of disease in cattle began in Mugla, Turkey. The disease was regarded as unusual or atypical for the region, and cases were reported to the Uludag University Faculty of Veterinary Medicine.

Similar reports were also received from Izmir, Canakkale, and Istanbul through the end of August 2007. The cattle had stomatitis, swelling of eyelids, respiratory distress, nasal and ocular discharge, redness and scaling of muzzle and lips, lameness, and udder erythema, and some were recumbent (Table 1). Body temperatures were elevated (39.7°C–41.1°C), except for 1 animal, whose temperature was 37.5°C, below the reference range for cattle (37.8°C–39.2°C). However, heart rates (mean 72 ± 3 beats/min) and respiratory rates (mean 24 ± 4 breaths/min) were within reference ranges of 60–80 beats/min and 10–30 breaths/min, respectively, for cattle with suspected disease. Cattle with EHD had tachycardia and tachypnea (Table 2). Causes of mucosal disease, stomatitis, and fever, including bovine viral diarrhea, foot and mouth disease, and infectious bovine rhinotracheitis, were considered, but the rate of spread and some of the clinical signs ruled out these diseases. However, the clinical signs of the disease were consistent with either EHD or BTV infection (*6,8–10*). These diseases were therefore considered as requiring further laboratory-based diagnostic assays.

A total of 41 blood samples were obtained from the affected cattle (35 Holsteins and 6 Brown Swiss, 2–5 years of age). Samples were obtained in tubes with and without EDTA. Complete blood analysis showed that 5 of the cattle with EHD had low leukocyte counts (online Appendix Table, available from www.cdc.gov/EID/content/15/2/317-appT.htm). After use for hematologic analysis, samples were stored at –30°C until virologic and serologic tests could be performed. Samples from the 41 animals were tested by ELISA for bovine viral diarrhea virus antigens; results were negative. To isolate virus, we spread unclotted blood samples onto baby hamster kidney–21 (BHK) cells.

Because EHDV had never been observed in Turkey, no diagnostic procedures were available. We therefore submitted selected samples (11 whole blood samples, 4 serum

Figure 1. Erosive lesion on pulvinus dentalis of cow seropositive for epizootic hemorrhagic disease virus, Turkey, 2007.
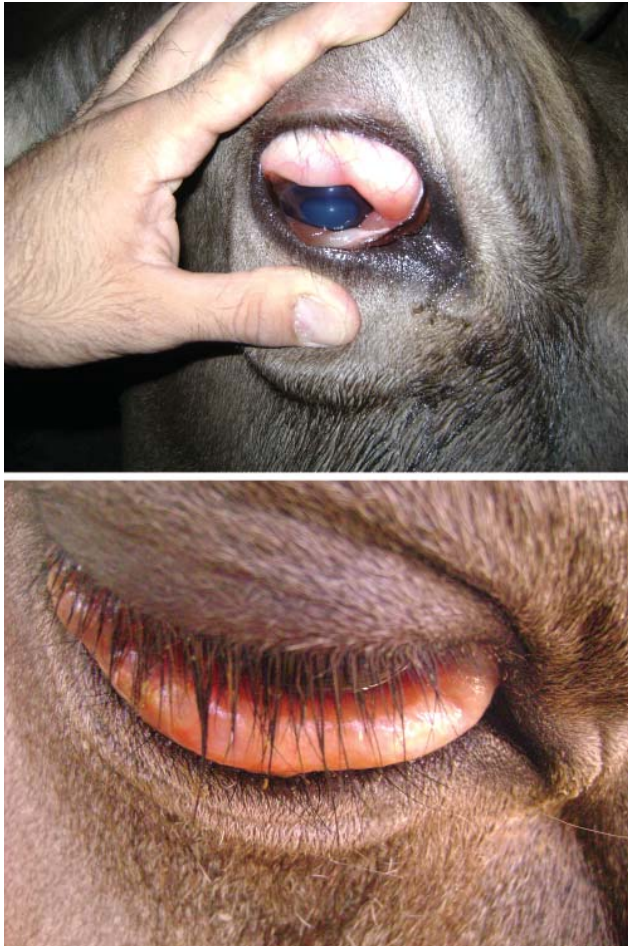
Figure 2. Swollen conjuctiva of cow seropositive for epizootic hemorrhagic disease virus, Turkey, 2007.

Mugla, was positive for EHDV; this cow died 3 hours after clinical examination and sample collection. The remaining cell culture supernatants were negative for EHDV. It is unusual to isolate EHDV by direct inoculation of BHK cells; initial passage through eggs or the *Culicoides variipennis* larvae cell line (KC cells) is usually required (*15*). The 4 serum samples were also tested for EHDV-specific antibodies by ELISA (*12*); only 1 sample was found to contain antibodies to EHDV.

Conventional RT-PCR of RNA extracted from the 11 original blood samples gave inconclusive results. Agarose gel electrophoresis indicated no product of the expected size. However, virus was isolated from 6 of the blood samples by using KC cells (dsRNA virus reference collection at the Institute for Animal Health, reference collection nos. TUR2007/01-06). These 6 samples and the 1 original positive cell culture were further tested by serotype-specific RT-PCRs that targeted segment 2 for identification of EHDV serotype. This analysis identified all viruses as EHDV-6, sharing 95.7% nucleotide sequence identity (segment 2, 110–670 bp) with the EHDV reference strain 318.

Conclusions

Of the selected samples submitted for BTV and EHDV testing, the positive identification of EHDV RNA supports initial clinical identification of an EHD outbreak in Turkey. The negative results from the blood samples may have resulted from degradation of viral RNA during transfer to the laboratory or insufficient sensitivity in the conventional RT-PCR. The propagation of another 6 virus isolates (TUR2007/01–06) by passage through KC cells indicates that virus was indeed present in the original blood samples, although not detected by conventional RT-PCR.

That only 1 of the 4 original serum samples was positive for EHDV antibodies by ELISA can be explained by time of sample collection. Antibodies to BTV can be detected from 8 days after infection (*11*); these samples may have been collected during the early stages of infection, before development of the immune response.

This study confirms EHDV infection of cattle in Turkey. EHD needs to be considered in the differential diagnosis of cattle with clinical signs that include fever; stomatitis; lameness; salivation; redness and scaling of the nose

samples, and 15 supernatant samples from the BHK cells) to the World Organisation for Animal Health reference laboratory for BTV (Institute for Animal Health, Pirbright, UK) for virologic and serologic analysis. All samples were tested for BTV by real-time RT-PCR and for EHDV by conventional RT-PCR (*13–15*). All results were negative for BTV. However, a conventional RT-PCR assay targeting genome segment 7 of EHDV (*15*) indicated that one of the cell culture supernatants, from an early case from

Table 1. Clinical signs in cattle tested for EHD, Turkey, 2007*

EHD status†	No. cattle with clinical sign									
	Discharge‡	Redness§	Recumbency	CE	Anorexia	RM	UE	Stomatitis	RD	Lame
Suspected (n = 41)	13	12	2	15	16	20	9	12	5	6
PCR+ (n = 1)	1	0	1	1	1	1	1	1	1	1
Seropositive (n = 1)	1	1	0	1	1	1	0	1	0	1
Virus isolated (n = 6)	5	6	0	4	6	6	0	4	3	3

*EHD, epizootic hemorrhagic disease; CE, conjunctival edema; RM, reduced milk; UE, udder edema; RD, respiratory distress.
†PCR, ELISA, and virus isolation were performed on selected samples from the 41 samples (11 whole blood samples, 4 serum samples, and 15 supernatant samples from the baby hamster kidney cells). The virus-positive animals were PCR negative.
‡Nasal and ocular discharge
§Redness and scaling of nose and lips.

Table 2. Vital signs of cattle tested for EHD, Turkey, 2007*

EHD status†	Temperature, °C	Heart rate, beats/min	Respiratory rate, breaths/min	Mucous membranes	Enlarged lymph nodes	Rumen motility, contractions/5 min
Suspected (n = 41)	37.5–39.2	72 ± 3‡	24 ± 4‡	Cyanotic (n = 2)	ND	0–12
PCR+ (n = 1)	40.5	110	52	Cyanotic	Prescapular, submandibular	0
Seropositive (n = 1)	41.1	104	48	Hyperemic	None	1
Virus isolated (n = 6)	39.7–40.6	68–86	32–56	Normal color	Submandibular (n = 1)	0–4

*EHD, epizootic hemorrhagic disease; ND, not detected.
†PCR, ELISA, and virus isolation were performed on selected samples from the 41 samples (11 whole blood samples, 4 serum samples, and 15 supernatant samples from the baby hamster kidney cells). The virus-positive animals were PCR negative.
‡Mean ± SEM.

and lips; swelling of the tongue; and erosions of the pulvinus dentalis, palatinum, and nose. More detailed studies of EHDV infection in cattle are needed.

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Natural Transmission of Zoonotic *Babesia* spp. by *Ixodes ricinus* Ticks

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To determine characteristics of natural transmission of *Babesia* sp. EU1 and *B. divergens* by adult *Ixodes ricinus* ticks, we examined tick salivary gland contents. We found that *I. ricinus* is a competent vector for EU1 and that their sporozoites directly invade erythrocytes. We conclude that EU1 is naturally transmitted by *I. ricinus*.

*I*xodes ricinus is a ubiquitous triphasic tick found commonly in Europe. This arthropod feeds on a wide variety of vertebrate hosts, including small rodents and wild and domestic ungulates. It is therefore a potential vector of numerous pathogens, such as bacteria, viruses, and parasites, mainly apicomplexans. Among these pathogens, 2 zoonotic *Babesia* species have been described in Europe: the well-known cattle parasite *Babesia divergens* (*1*) and the more recently reported roe deer parasite *Babesia* sp. EU1 (*2–4*). Biological transmission of *B. divergens* by *I. ricinus* ticks has been proven by in vivo experimental infections (*5*); however, quantitative transmission studies that visualize and quantify sporozoites have never been conducted. For *Babesia* sp. EU1, biological evidence of natural transmission by *I. ricinus* ticks is still lacking; its presence has been assessed only by DNA amplification from whole ticks (*4,6–8*). Therefore, to analyze transmission of zoonotic *Babesia* spp. by *I. ricinus* ticks, we visualized, isolated, and identified infectious sporozoites from dissected tick salivary glands, the transmitting organs.

The Study

In 2008, ticks were collected from animals from 2 different biotopes where each *Babesia* species had been known

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to circulate: a farm on which a herd was infected with *B. divergens* and a reserve on which wild fauna were infected with *Babesia* sp. EU1. A dairy farm in La Verrie (Vendée, France) was selected as a favorable biotope for *B. divergens* transmission on the basis not only of the presence of numerous ticks on cows and in pastures in 2007 but also of the parasite circulation in the herd, attested by serologic testing (prevalence of 37.5% by immunofluorescence antibody test [IFAT]) and confirmed by its isolation from cattle erythrocytes (prevalence 25% by culture) (*9*). Of the cows tested by IFAT, 56% had positive results, which indicated that new infections from ticks were occurring within the herd. Because we assumed that sporozoite differentiation is stimulated by blood ingestion and because of experimental proof that female ticks can transmit *B. divergens* (*10*), we collected only adult ticks feeding on cows. The 324 collected ticks were morphologically identified as *I. ricinus* and weighed to estimate their repletion status (range 3–398 mg). Of these, 223 ticks (4.7–339 mg) were dissected under a stereomicroscope to isolate both salivary glands, which were subsequently crushed in 30 µL phosphate-buffered saline in a 1.5-mL microtube with an adapted pestle. A droplet of this suspension was deposited on an 18-well slide, stained with May-Grünwald-Giemsa, and examined under a light microscope. When parasites were seen, and for 41 additional negative samples within the same weight range, 5 µL of the infected suspension was added to the culture medium with bovine (*B. divergens* selective growth) or sheep (both species growth) erythrocytes, RPMI (Roswell Park Memorial Institute medium; Lonza, Basel, Switzerland), and 20% fetal calf serum (Lonza) in 96-well plates (*11*).

To identify the parasites, we directly sequenced the amplified 18S rDNA *Babesia* gene. PCR with Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was performed on extracted DNA (Wizard Genomic DNA Purification Kit; Promega, Madison, WI, USA) from the remaining crushed salivary gland suspensions (Bab primers GF2 and GR2, 540 bases long, variable part of the gene) (*4*) and from resulting parasitized erythrocytes (primers CryptoF and CryptoR, 1,727 bases long, complete gene) (*12*).

To confirm the identity of the infected ticks, we directly sequenced a variable part of the 16S rDNA mitochondrial gene of *Ixodes* ticks (310 bases long) (primers IrUp1 5′-TTGCTGTGGTATTTTGACTATAC-3′ and IrDo2 5′-AATTATTACGCTGTTATCCCTGA-3′). We used DNA extracted from salivary glands.

Microscopic observation of crushed salivary gland suspensions identified small pear-shaped elements in only 3 ticks; weights were 11.7, 25.3, and 277 mg. These millions of pyriform parasites were considered to be sporozoites (*13*): they measured about 2 µm in length and 1 µm in diameter (Figure, panel A). Only a few parasites had unusual forms, which suggests binary fission (Figure, panel B).
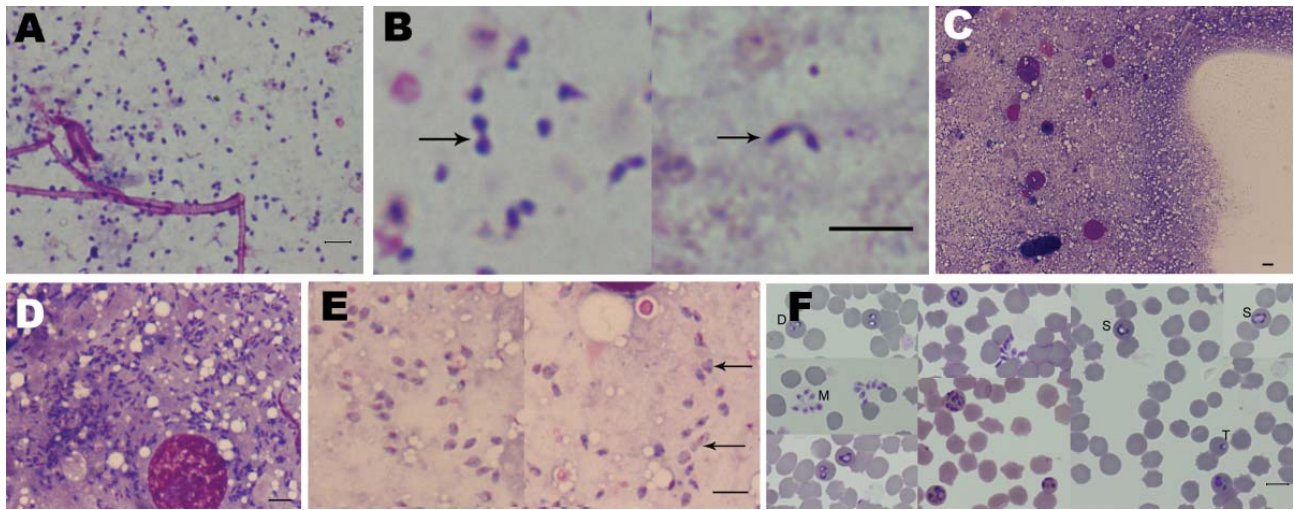
Figure. Microscopic appearance of *Babesia* sp. EU1 sporozoites isolated from tick salivary glands and of subsequent asexual development in erythrocytes. Sporozoites were stained with Giemsa and observed in the suspension of crushed salivary glands (A, B) and from salivary glands directly crushed between slides (C, D, E). Arrows indicate sporozoite dividing forms. A composite panel of asexual stages cultivated in sheep erythrocytes from these sporozoites is presented (F); developmental stages are indicated by letters (D, dividing stages; M, free merozoites; S, schizont-like form; T, trophozoite). Scale bars = 5 μm.

Development of intraerythrocytic parasites was observed, which proved the parasites' capacity to directly infect erythrocytes. Of the 3 tick salivary glands containing pear-shaped elements, 3 days after inoculation onto a culture, ≈1/10,000 erythrocytes was infected. Only sheep erythrocytes were invaded, which suggests infection with *Babesia* sp. EU1. From these 150-μL starting wells, 10-mL amplified cultures (10% parasitized erythrocytes) could be established within 1 month (Figure, panel F). Typical *Babesiidae* developmental forms (trophozoite, dividing stages, and free merozoites) were observed, as were more atypical schizont-like parasites, which seemed to produce numerous merozoites. When sporozoites were not observed, parasites were never observed in the cultures of either bovine or sheep erythrocytes.

PCR amplification, sequencing, and comparison with *Babesia* spp. 18S rDNA genes (BLAST [www.ncbi.nlm.nih.gov/blast/Blast.cgi] search in GenBank) showed the sequences to be 100% identical to the *Babesia* sp. EU1 sequence (AY046575) for the 3 infected ticks (sporozoites and culture). The partial (sporozoites) and complete (culture) 18S rDNA sequences obtained have been deposited in GenBank, accession nos. FJ215872 and FJ215873. Identity of the ticks was confirmed by sequence analysis and comparison with the 16S rDNA *I. ricinus* gene (U14154).

For the wild fauna reserve, we used the same approach. At the reserve of Chizé (Deux-Sèvres, France), where high prevalence *Babesia* sp. EU1 has been described (*4*), we captured 18 roe deer, then collected and analyzed blood samples from them. Presence of *Babesia* sp. EU1 was attested by culture of samples from 4 of the deer. For 31 female ticks, half of the ticks were processed as previously described, and the salivary glands of the other half were simply crushed between 2 slides so parasites could be better seen and quantified. With the latter method, a huge number of sporozoites, ≈10^7 to 10^8, were observed (Figure, panels C, D). The inner structures were well preserved, nuclei were clearly visible, and we could observe apparent dividing forms (Figure, panel E). From the ticks collected from roe deer, only 2 tick salivary glands contained parasites; PCR products using Bab primers showed 100% identity with *Babesia* sp. EU1 (AY046575).

Conclusions

Our study shows that *I. ricinus* ticks are competent vectors for *Babesia* sp. EU1. Not only can these ticks carry *Babesia* sp. EU1 DNA, but more importantly, they enable these parasites to complete their life cycle up to the production of infectious sporozoites. Direct invasion of erythrocytes by *Babesia* sp. EU1 undoubtedly classifies this species in the genus *Babesia*, a feature generally not proven for most *Babesia* spp.

The proportions of *Babesia* sp. EU1–infective ticks found in our study (3/223 from cattle farm and 2/31 from wild fauna reserve, not statistically different) are comparable to published prevalence of infected ticks (1%–2%) collected either from animals or vegetation (*6–8,14,15*). Whatever the biotope, *Babesia* sp. EU1 is always present, threatening also in anthropized zones (farming areas). Millions of parasites inside salivary glands were observed and could be injected to the vertebrate host, from the early stage of the tick feeding (11.7 mg) until repletion (277 mg),

which represents a massive infection. These 2 epidemiologic features, combined with the increasing number of immunocompromised persons, should lead to more awareness of the risk related to this zoonotic pathogen.

B. divergens sporozoites were never seen in the salivary glands of adult *I. ricinus* ticks, even when ticks were collected from cattle. This finding is despite the large number of ticks examined (223), the prevalence of nymphs carrying *B. divergens* DNA collected from the farm pastures (87% in 2007 on 113 nymphs analyzed, data not shown), and the infectious status of the herd (serologic prevalence 56%). We therefore raise questions about the main transmitting stage (larvae, nymph, or adult?) and about the quantitative transmission of *B. divergens* by *I. ricinus* ticks (low number of produced and infectious sporozoites?). In Europe, human babesiosis could be caused by these 2 *Babesia* spp., each of which is transmitted by *I. ricinus* ticks but probably with different sporozoite-production features.

Mrs Becker is a veterinarian. She is pursuing a doctorate degree at the Institut National de la Recherche Agronomique, Département de Santé Animale. Her main research interests are apicomplexan pathogens, specifically with respect to their transmission and human and veterinary health importance.

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Venezuelan Equine Encephalitis and Upper Gastrointestinal Bleeding in Child

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Venezuelan equine encephalitis (VEE) is reemerging in Peru. VEE virus subtype ID in Peru has not been previously associated with severe disease manifestations. In 2006, VEE virus subtype ID was isolated from a boy with severe febrile disease and gastrointestinal bleeding; the strain contained 2 mutations within the PE2 region.

Venezuelan equine encephalitis (VEE) is a reemerging disease in the Amazon region of Peru; subtype ID is the subtype most commonly isolated from humans (*1–3*). Although the typical clinical presentations of VEE are neurologic and febrile syndromes, in the Amazon region, the common presentation is a febrile syndrome with mild or no neurologic involvement (*1–4*). Human infections with VEE virus subtype IAB and IC produce more neurologic involvement and result in mortality rates as high as 0.5% during epidemics; however, epidemics with these viruses have not been reported in South America for more than a decade (*5–7*). During past epidemics, most neurologic disease and fatal cases were in children and elderly persons (*8,9*).

In 2006, an outbreak of febrile disease occurred in Jeberos, a rural Amazonian community in Loreto Peru, located 14 hours, by river, from Yurimaguas, Peru (Figure 1). Although VEE cases had been reported previously in Yurimaguas (T. Kochel, P.V. Aguilar, unpub. data), they had not been reported in Jeberos. We describe here the clinical manifestation of VEE subtype ID in a boy from Jeberos. The boy had severe disease; upper gastrointestinal bleeding; and neurologic, renal, and liver complications. He responded to supportive therapy.

The Case

On January 9, 2006, a 3-year-old boy from Jeberos was admitted to the Hospital Santa Gema in Yurimaguas. Three days before hospitalization, he had fever, chills, and malaise. Two days before hospitalization, the symptoms persisted and the boy was brought by his parents to the Health Center of Jeberos, where he received antipyretic medication (acetaminophen and the nonsteroidal antiinflammatory drug metamizole) for fever (40°C). One day before hospitalization, the boy became sleepy and irritable and had 2 episodes of vomiting followed by melena. The boy was then transferred by airplane to the Hospital Santa Gema in Yurimaguas. On the day of hospitalization, the boy was in poor general condition and had coffee-ground emesis (with blood clots) and melena. His temperature was 37ºC, blood pressure was 90/60 mm Hg, respiratory rate was 45 breaths/min, and heart rate was 132 beats/min.

Figure 1. Map of Peru showing Jeberos community in Yurimaguas, Loreto.

He became somnolent, and marked paleness and scarce petechiae on both legs and severe dehydration were noted. No signs of jaundice, lymphadenopathy, or conjunctival bleeding were observed. No other signs were found in the thorax or cardiovascular system. Focal or meningeal signs and joint inflammation were absent.

Laboratory test results were as follows: leukocytes 12,200/mm³ (22% segmented cells, 0% bands, 75% lymphocytes, and 2% monocytes); platelets 122,000/mm³, hemoglobin 56 g/L, and hematocrit 17%. Liver function was altered: alanine aminotransferase (ALT) 132 U/L, aspartate aminotransferase (AST) 140 U/L, serum albumin 2.1 g/dL, and total bilirubin 2.4 mg/dL (direct bilirubin 1.4 mg/dL). Serum creatinine was 2.2 mg/dL and, because of the boy's age, was considered acute renal failure. Preliminary diagnoses were febrile and hemorrhagic syndrome with severe dehydration and anemia. On the day of admission, January 9, a blood sample was sent to the Naval Medical Research Center Detachment in Lima, Peru, and the National Institute of Health–Ministry of Health of Peru, for advanced analysis.

The boy was given supportive therapy, antimicrobial drugs, and blood replacement (200 mL). On January 10, his leukocyte count had dropped to 4,200/mm³ (26% segmented, 14% bands, 56% lymphocytes, and 2% monocytes). After the transfusion, his hematocrit was 25%; his platelet count was 108,000/mm³ in the morning and decreased to 60,000/mm³ by night (Table). Test results for malaria (blood smear), agglutinins for typhoid fever, and surface antigen of hepatitis B virus were negative. The boy was hydrated and afebrile, but the melena and coffee-ground vomiting (with blood clots) persisted. On January 11, his hematocrit was 20% and platelet count was 91,000/mm³; he was still lethargic and the gastrointestinal bleeding persisted. On January 12, he received a transfusion of 150 mL whole blood; however, he still showed neurologic involvement (e.g., drowsiness, lethargy, confusion) and neck stiffness. On January 16, the boy's condition improved and the gastrointestinal bleeding stopped. Liver and renal functions returned to within normal limits: serum creatinine

1.6 mg/dL, ALT 40 U/L, AST 18 U/L, urine volume 535 mL/24 h, and urine protein 36 mg/24 h. The boy's mental status improved progressively, and he was discharged on January 20.

VEE virus was isolated from serum culture in Vero cells. No other viruses were isolated in Vero or C6/36 cells. A convalescent-phase sample obtained ≈2 weeks after symptom onset had a >4-fold higher titer than the acute-phase sample, according to a previously described immunoglobulin (Ig) M ELISA specific for VEE (2). The sample was IgM negative for other local arthropod-borne viruses such as dengue, yellow fever, Mayaro, Oropouche, and members of the group C complex. Genetic analyses using previously described methods further identified the VEE strain as subtype ID, genotype Panama-Peru (1,4) (Figure 2). The strain was genetically similar to other strains isolated previously in Peru; however, 2 amino acid changes were observed within the envelope glycoprotein precursor (PE2) region (H→Q, V→I). The National Institute of Health–Ministry of Health reported negative results for leptospirosis and rickettsial diseases, according to IgM ELISA and indirect immunofluorescent assay, respectively.

In February 2006, another case of VEE in a child was reported. A 10-year-old boy with similar clinical signs, including headache, vomiting, melena, and altered mental status (somnolence and confusion) was admitted to the hospital in Yurimaguas. The boy, a resident of Esperanza village, Lagunas district, had visited the Aypena River near Jeberos. The acute-phase serum sample was positive for VEE virus by PCR; however, no virus was isolated in cell culture. A convalescent-phase sample, taken a week after onset of signs, was negative by VEE IgM ELISA; no additional convalescent-phase sample was obtained from the patient. No epizootics were reported.

Conclusions

VEE virus subtype ID was isolated from a child with neurologic and severe hemorrhagic manifestations in the northern Peruvian jungle. During this episode, local health workers in Jeberos reported that they had evaluated at least

Table. Blood cell counts and hemoglobin values in a 3-year-old boy with Venezuelan equine encephalitis, Jeberos, Peru, January 2006*

| Date/time of sample collection	Platelets/ mm³	Hematocrit, %	Leukocytes/ mm³	Leukocytes, %					Hemoglobin, g/L
				Neutrophils		Eosinophils	Monocytes	Lymphocytes	
				Bands	Segmented				
Jan 9	122,000	17	12,900	0	22	2	2	76	56
Jan 10									
4 h	108,000	25	4,200	14	26	2	2	56	
10 h	120,000	23	NM	NM	NM	NM	NM	NM	NM
16 h	87,000	22	NM	NM	NM	NM	NM	NM	NM
22 h	60,000	21	NM	NM	NM	NM	NM	NM	NM
Jan 11	91,000	20	NM	NM	NM	NM	NM	NM	NM
Jan 16	230,000	35	8,900	2	70	NM	NM	28	NM

*NM, not measured.
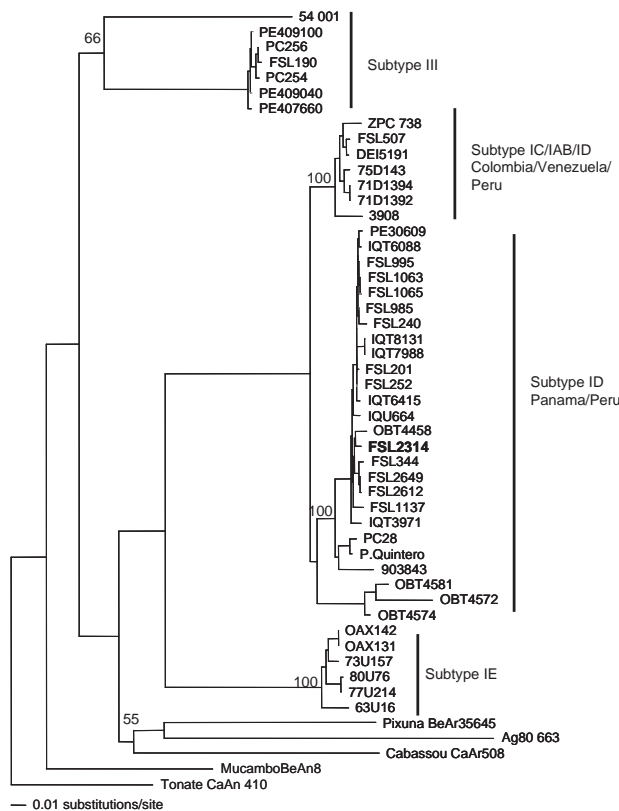
Figure 2. Neighbor-joining phylogenetic tree of Venezuelan equine encephalitis virus complex based on partial sequence of the envelope glycoprotein precursor segment. The strain isolated from the 3-year-old boy with upper gastrointestinal bleeding, Jeberos, Peru, January 2006, is shown in **boldface**. Numbers indicate bootstrap values.

5 cases with some hemorrhagic manifestations; however, no samples were obtained from those cases, and thus there is no definitive proof that the patients were infected with VEE virus.

The VEE strain isolated from the boy with confirmed VEE contained 2 mutations within the PE2 region, which differed from other subtype ID strains from Peru. Nevertheless, the contribution of these or any other possible mutations in the viral genome to the hemorrhagic manifestation observed in the patient remains unknown. Many questions remain about the effect of VEE virus in the Jeberos community.

Acknowledgments

Dr Vilcarromero is a physician who responds to outbreaks and individual cases in Yurimaguas, Peru, and provides healthcare in the Hospital Santa Gema. He participates in the "Surveillance and Etiology of Acute Febrile Illnesses in Peru" project, carried out by the Naval Medical Research Center Detachment, Ministry of Health, and the Cayetano Heredia and San Marcos universities in Peru.

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Isolation of Kyasanur Forest Disease Virus from Febrile Patient, Yunnan, China

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We recently determined that Nanjianyin virus, isolated from serum of a patient in Yunnan Province, China, in 1989, is a type of Kyasanur Forest disease virus. Results of a 1987–1990 seroepidemiologic investigation in Yunnan Province had shown that residents of the Hengduan Mountain region of had been infected with Nanjianyin virus.

Kyasanur Forest disease (KFD) virus, a member of the tick-borne encephalitis virus serocomplex of the genus *Flavivirus*, family *Flaviviridae*, can cause fever, hemorrhage, and encephalitis and has a 3%–5% case-fatality ratio (*1*). KFD was discovered in 1957 in the Mysore forest region of south India, where 400–500 persons per year were infected with the virus (*2,3*). KFD virus has been found only in monkeys, humans, and *Haemaphysalis spinigera* ticks in the KFD-epidemic region of south India (*4*), although a variant of KFD virus, Alkhurma virus, was isolated recently in Saudi Arabia (*5*). In this study, we determined that the gene sequence of a Nanjianyin virus isolate obtained from a febrile patient is highly homologous to that of KFD virus. The Nanjianyin virus was isolated in 1989 from the serum of a 38-year-old woman from the Hengduan Mountain region of Yunnan Province, People's Republic of China, where a previous serosurvey demonstrated that KFD exposure had occurred (Figure 1).

The Study

In tests conducted shortly after isolation of Nanjianyin virus in 1989, the virus caused a typical cytopathic effect within 4 days after its injection in BHK-21 cells, killed 100% of 3-day-old mice within 2.5 days after their intrac-

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erebral inoculation with a 25-μL culture supernatant, and killed 100% of 50-day-old adult mice within 11–13 days of their intraperitoneal inoculation with a 30-μL culture supernatant. Hemagglutination inhibition test results showing a cross-reaction between Nanjinayin virus and a Japanese encephalitis virus antibody indicated that Nanjianyin virus belonged to the genus *Flavivirus*. No further tests to classify Nanjianyin virus were performed at the time it was isolated. The virus was preserved by lyophilization and stored at –30°C.

Recently, we used molecular methods to determine that Nanjianyin virus is a variant of KFD virus. After reconstituting the lyophilized virus in a BioSafety Level 3 biosafety cabinet, we suspended the sample in 0.5 mL minimum essential media (Gibcol BRL, Gaithersburg, MD, USA) (pH 7.4) and then centrifuged it for 5 min at 6,000× *g*. We then extracted the total RNA from 140 μL of supernatant by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's protocol and produced the first strands of cDNA by using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Piscatawy, NJ, USA) as described in the manual accompanying the kit. We used *Flavivirus* genus-specific primers (*6*) to perform reverse transcription-PCR amplification using viral genomic RNA as a template and determined the nucleotide sequence of the virus from the amplified cDNA fragment. Results of nucleotide sequence analysis by BLAST (http://blast.ncbi.

Figure 1. Maps showing location of Yunnan Province in China (red) and counties in the Hengduan Mountain region of Yunnan Province where Kyasanur Forest disease virus antibody has been detected. 1, Lushuii County, antibody found in 31.6% of humans, 25.5% of birds, and 15.4% of rodents; 2, Yingjiang County, antibody found in 46.7% of humans; 3, Longchuang County, antibody found in 6.4% of humans; 4, Ruili County, antibody found in 7.7% of humans; 5, Mangshi County, antibody found in 32.5% of humans; 6, Shidan County, antibody found in 6.3% of humans; 7, Nanjian County, county in which Nanjianyin virus was found in 1989; 8, Eryuan County, antibody found in 4.9% of birds; 9, Lijiang County, antibody found in 0.7% of humans; 10, Shangri-La County, antibody found in 8.5% of humans.

nlm.nih.gov/Blast.cgi) showed that the nucleotide DNA sequence of Nanjianyin virus was 99% homologous to that of KFD virus (prototype KFDV *Itp9605,* GenBank accession no. AY323490).

To complete the sequence determination of the PrM-E genes, we designed 3 pairs of primers to amplify them. Using information from a previous study (*6*), we also designed an additional primer pair to amplify the nonstructural protein (NS5) gene (Table).

Results of sequence alignment and homology analysis performed with MegAlign software of DNASTAR (Madison, WI, USA) showed that the 654-bp PrM gene of Nanjianyin virus was 99.6% identical to that of KFD virus (Itp9605 strain), 99.4% identical to that of KFD virus (EU480489), 98.2% identical to that of KFD virus (X74111), but only 90.4% identical to that of Alkhurma hemorrhagic fever (AFH) virus (1176 strain), and only 57.2% to 64.3% identical to the 654-bp PrM genes of other tick-borne encephalitis complex viruses such as Omsk hemorrhagic fever virus (Kubrin strain), tick-borne encephalitis virus (Sengzhang strain), Powassan virus (LB strain), and Langat virus (TP21 strain). The 1,487-bp E gene nucleotide sequence of Nanjianyin virus was 99.8% identical to that of KFD virus (Itp9605 strain), 99.8% identical to that of KFD virus (EU480489), 98.5.0% identical to that of KFD virus (X74111), 91.9% identical to that of AFH virus (1176 strain), and <72% identical to that of other tick-borne encephalitis complex viruses. The nucleotide sequence of the 1,000-bp NS5 gene of Nanjianyin virus was 99.6%, 99.7%, and 99.7% homologous to that of KFD virus (Itp9605 strain), KFD virus (W371), and KFD virus (EU480489), respectively; 92.3% homologous to that of AFH virus isolate 1176; and <77.6% homologous to the 1,000-bp NS5 gene of other tick-borne encephalitis complex viruses. Results of homology analyses thus demonstrated that Nanjianyin virus belongs to the KFD virus clade, and results of phylogenetic analyses conducted with 2,142 nt of the PrM-E gene and 1,000 nt of the NS5 gene suggested that Nanjianyin virus and KFD virus are in the same genetic cluster (Figure 2).

Conclusions

Results of a serosurvey of tick-borne viruses conducted from 1987 through 1990 in Yunnan Province (*7*) showed that 169 (19.5%) of 867 healthy residents of western Yunnan Province (in Lushui, Shidian, Yingjiang, Mangshi, Ruili, and Longchuan counties) and 6 (3.7%) of 161 healthy residents of northwestern Yunnan Province (in Lijiang and Diqin counties) carried antibodies against KFD virus. KFD antibodies also were detected in the serum of patients with fever in Lushui County (*7,8*) and in the serum of resident birds, migratory birds, rodents, and rhesus monkeys (*Macaca mulatta*) in the Hengduan Mountain region

Table. Primers used to sequence the PrM-E and NS5 genes of Nanjianyin virus*

Primers	Primer sequence (5′ → 3′)
PrM-E gene primers	
KFD1F(105-124)	CGGACTGGTATTGATGCG
KFD1R(1357-1340)	TCTTCTCGGACTGCGTTG
KFD2F(1100-1117)	ACCAGGCGAGCACAGTCT
KFD2R(1952-1935)	CCTCCTCCAGTTGTTTCCA
KFD3F(1652-1673)	GAGTGCCCGTGGCTAACATAGA
KFD3R(2832-2812)	CTTGGTCCTCATTCCCATCCC
NS5 gene primers	
FU1PM (8908-8933)	TACAACATGATGGGVAARAGWGA RAA
cFD3 (9961-9983)	AGCATGTCTTCCGTGGTCATCCA

*NS, nonstructural protein.

(Lushui and Eryuan counties) (*7,9*). These results indicate that humans and animals in the Hengduan Mountain region of Yunnan Province have been infected with KFD virus since the 1980s. Although detailed information about the movement of the woman infected with Nanjianyin virus in 1989 is not available, residents of the Hengduan Mountain region at that time seldom traveled far, so she probably was
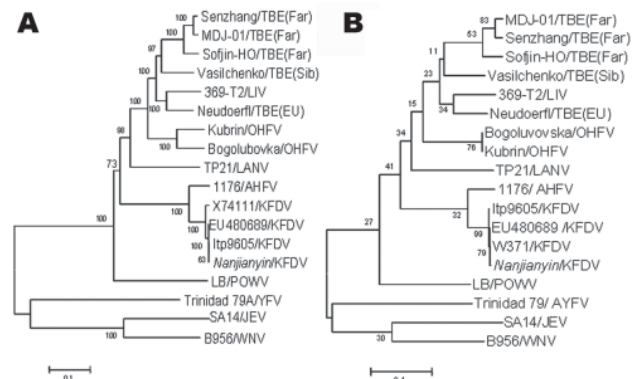

Figure 2. Phylogenetic analysis of the PrM-E (A) and nonstructural protein 5 (B) gene sequences of Nanjianyin virus isolated from Yunnan Province, China. Phylogenetic analyses were performed by the neighbor-joining method with MEGA version 3.1 software (www.megasoftware.net). Bootstrap probabilities of each node were calculated with 500 replicates. Scale bars indicate number of nucleotide substitutions per site. Abbreviations and GenBank accession numbers are as follows: tick-borne encephalitis virus (TBEV), TBEV (Far), strain MDJ-01(AY217093); TBEV (Far), strain Senzhang (AY182009); TBEV (Far), strain Sofjin-HO (AB062064); TBEV (Sib), strain Vasilchenko (AF069066); louping iII virus (LIV), strain 369-T2 (Y07863); TBEV (EU), strain Neudoerfl (U27495); Omsk hemorrhagic fever virus (OHFV), strain Bogoluvovska (AY193805); OHFV, strain Kubrin (AY438626); Langat virus (LANV), strain TP21 (AF253419); Alkhurma hemorrhagic fever virus (AHFV), strain 1176 (AF331718); Kyasanur Forest disease virus (KFDV), strain unknown (PrM-E, X74111); KFDV, strain unknown (EU480689); KFDV, strain Itp6905 (AY323490); KFDV, strain W371 (NS5, AF013385); KFDV, strain Nanjianyin (PrM-E, EU918175; NS5, EU918174); Powassan virus (POWV), strain LB (L06436); yellow fever virus (YFV), strain Trinidad 79 (AF094612); Japanese encephalitis virus (JEV), strain SA14 (D90194); West Nile virus (WNV), strain B956 (AY532665).

exposed there.

Results of epidemiologic and virologic investigations suggest that migratory birds play a key role in the spread of arboviruses (*10,11*). Migratory birds frequently pass through Yunnan Province during their migration from south India and the Indian Ocean islands to Mongolia and Siberia. The areas adjacent to Hengduan Mountain in Yunnan Province and India also provide a suitable habitat for *Haemaphysalis spinigera,* which is the vector for KFD virus in the region (*12,13*). Our results, combined with those in previous seroprevalence reports of KFD virus in humans and birds (*6,7*), indicate that KFD virus likely was carried to the region by these migratory birds and their parasitic ticks. KFD antibodies have been detected in residents of north and northeast India, and the KFD seropositive rate is especially high among residents of India's Andaman Islands and Nicobar Islands (*14*). KFD antibodies also were detected in both human and bird serum in the Chinese districts of Guangdong, Guangxi, Guizhou, Hubei, Henan, Xinjiang, and Qinghai in 1983 (*15*).

In summary, we found that Nanjianyin virus, first isolated in the Hengduan Mountain region of Yunnan Province, is a variant of KFD virus. This finding confirms that infection with KFD virus has previously occurred in the region and justifies enhanced surveillance for KFD among febrile patients in the Hengduan Mountain region.

Acknowledgments

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Chikungunya Virus and Central Nervous System Infections in Children, India

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Ravi Ravikumar, and Tom Solomon

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus best known for causing fever, rash, arthralgia, and occasional neurologic disease. By using real-time reverse transcription–PCR, we detected CHIKV in plasma samples of 8 (14%) of 58 children with suspected central nervous system infection in Bellary, India. CHIKV was also detected in the cerebrospinal fluid of 3 children.

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that causes illness characterized by fever, rash, and severe arthralgia. It was first described in Africa in 1952, and outbreaks occurred in India in the 1960s and early 1970s (*1*). Neurologic complications were reported occasionally (*2,3*). In 2005, an epidemic of CHIKV disease occurred among the populations of Réunion and other Indian Ocean islands (*1,4*), and spread to India by early 2006, where an estimated 1.3 million persons were infected (*5,6*). During a prospective study of all children with suspected central nervous system (CNS) infections admitted to a hospital in rural southern India, we noticed an unseasonal increase in admissions. This increase occurred at the same time as the CHIKV outbreak in southern India, so we investigated our cohort for CHIKV infection.

The Study

From January through October 2006, we studied children (≤16 years of age) admitted to the pediatric department of the Vijayanagar Institute of Medical Sciences, Bellary,

India, with suspected acute CNS infection. Acute CNS infections were suspected in those children with a febrile illness (<2 weeks' duration) and 1 of the following signs or symptoms: meningism, photophobia, severe headache, altered mental status, seizures, or focal neurologic signs. Children with previous neurologic conditions or *Plasmodium falciparum* malaria were excluded. The study was approved by the ethical committees of the hospital, the Indian Council for Medical Research, and the University of Liverpool, United Kingdom. Informed consent was obtained from the accompanying parent or guardian.

A detailed history was taken, and a neurologic examination was performed by a member of the study team. Routine blood samples were collected, and a lumbar puncture was performed. To detect CHIKV RNA in the plasma or cerebrospinal fluid (CSF), real-time reverse transcription–PCR for a 127-base region of the envelope E1 gene was performed (*7*). The E1 gene was subsequently amplified by RT-PCR, and sequenced (*7*). A 529-base region of the sequence was aligned with other CHIKV E1 gene sequences by using Lasergene software (DNASTAR, Inc., Madison, WI, USA), and phylogenetic analysis was performed on the align sequences by using Mega 4 software (*8*). To detect antibodies against Japanese encephalitis (JE) virus and dengue virus, which circulate in this area, serum specimens and CSF were tested by immunoglobulin M (IgM) capture ELISA (*9*). PCR for JE virus was also performed on CSF samples (*10*).

From January 1 through October 31, 2006, 66 children were recruited for the study; 37 (56%) were male, median age was 7 years (range 8 months–16 years); 58 had at least 1 plasma sample, and 57 had a CSF sample available for testing. CHIKV was detected in 8 (14%) of the 58 plasma samples and in 3 (5%) of the CSF samples; CHIKV was not detected in 2 CSF samples, and no CSF samples were available for 3 children. The median (range 2–23) of days for a positive plasma sample was 3.5 days; the 3 positive CSF samples were all obtained within 4 days of illness onset (online Appendix Table, available from www.cdc.gov/EID/content/15/2/329-appT.htm). Samples from all 8 patients were negative for malaria parasites and JE and dengue IgM antibodies. We also tested samples obtained before the outbreak (October 2005 through December 2005) and after the outbreak (November 2006 through December 2007); all were CHIKV negative.

Of the CHIKV-positive children, 7 children had altered mental status, which was associated with seizures in 6 patients; 3 children with both altered mental status and seizures also had meningism. Two children had a rash when they were hospitalized, and a rash developed in a third child on day 5 of hospitalization. Seven children had seizures and 4 had status epilepticus (seizure >30 min). Three children were aphasic and had extensor plantar reflexes. One 9-year-

old girl (patient 5) had experienced 2 days of fever, vomiting, and a generalized tonic-clonic seizure at home that lasted 3 minutes; this occurred 3 days after she received a live attenuated SA14-14-2 JE vaccine. When hospitalized, she had a score of 15 on the Glasgow Coma Scale (GCS) and was monitored without a lumbar puncture. CHIKV was detected in her plasma. Only 2 of the 5 patients whose CSF was analyzed had pleocytosis (>5 cells $\times$ 10^9/mL). Two children had reduced GCS scores, and 1 child remained aphasic when discharged. The other 6 patients were discharged with a full GCS score. At her 4-month follow-up visit, patient 8, who had CHIKV detected in her CSF and plasma, was performing poorly at school and had back and joint aches.

An 8-month-old girl (patient 7) was admitted who had experienced a fever for 7 days, multiple seizures, a widespread rash, and loss of appetite. She also had reduced hearing, a GCS score of 13, a vacant stare, frequent blinking, hepatomegaly (4 cm), and splenomegaly (6 cm). While she was an inpatient, gangrene developed in her fingers and toes (Figure 1). Her initial plasma and CSF samples were all used for clinical management, but a subsequent plasma sample was positive for CHIKV on day 23 of illness.

E1 gene PCR products sufficient for sequencing were amplified from plasma samples of 5 patients and the CSF of 1 patient. Sequences were deposited in GenBank under accession nos. EU856107–EU856112. Sequences were identical except for one that had a single nucleotide change from A to G at position 10625, resulting in an amino acid change from lysine to arginine at residue 211 of the E1 protein. The sequences from our cohort all had an alanine residue at position 226 of the E1 protein. This finding is typical of 90% of viral sequences from the Réunion Islands from June 2005 through October 2005 (*11*). Isolates from the Réunion Island outbreak all had a valine at this position (*11*). Scientists have postulated that the change at E1–A226V may be important for adaption to the mosquito vector, *Aedes albopictus,* and for neurovirulence (*11–13*). Our isolates lack this substitution. Phylogenetic analysis showed that the viruses were more closely related to those from the recent Indian Ocean CHIKV outbreaks and East African strains than to the Asian strains endemic in the region (Figure 2).

Conclusions

Our study has confirmed that during a CHIKV outbreak, the virus may be an important cause of neurologic disorders in children. Recent studies have described a wide range of neurologic manifestations, including meningoencephalitis, seizures, and Guillain-Barré syndrome (*14–16*). Our study shows that CHIKV is a likely cause of CNS infection. During the outbreak period from January 2006 through October 2006, we found that CHIKV was responsible for 14% of suspected CNS infections. In 1 of our patients, an 8-month-old girl for whom no acute-phase sample was available, the virus was detected at day 23 of illness, an unusually persistent level of viremia. The severity of her illness, with marked rash, hepatosplenomgaly, and digital gangrene could have been due to her inability to clear the virus. Alternatively, she may have become infected with CHIKV during her hospital stay. If one excludes this patient from the analysis, CHIKV was detected in the plasma and CSF samples of 10% of patients with suspected CNS infection. Some children had other features suggestive of CHIKV infection, but in 4 case-patients, only neurologic symptoms were present. Notably, 1 child had received JE vaccine 3 days before admission as part of a mass JE vaccination campaign in India (*17*). Her illness was attributed to an adverse event after vaccination (*18*). We demonstrated that her illness was equally coincident with CHIKV infection, illustrating the importance of thorough investigation of cases of adverse events after vaccination.

In our study, we chose to rely on PCR detection of the virus to diagnose CHIKV infection rather than testing for IgM antibodies, which may persist for several months after infection and could reflect coincidental infection (*19*) rather than an acute infection. In summary, during CHIKV outbreaks, clinicians should be aware that CHIKV may be a cause of CNS infections among children.

Figure 1. Digital gangrene in an 8-month-old girl during week 3 of hospitalization. She was admitted to the hospital with fever, multiple seizures, and a widespread rash; chikungunya virus was detected in her plasma. A) Little finger of the left hand; B) index finger of the right hand; and C) 4 toes on the right foot.
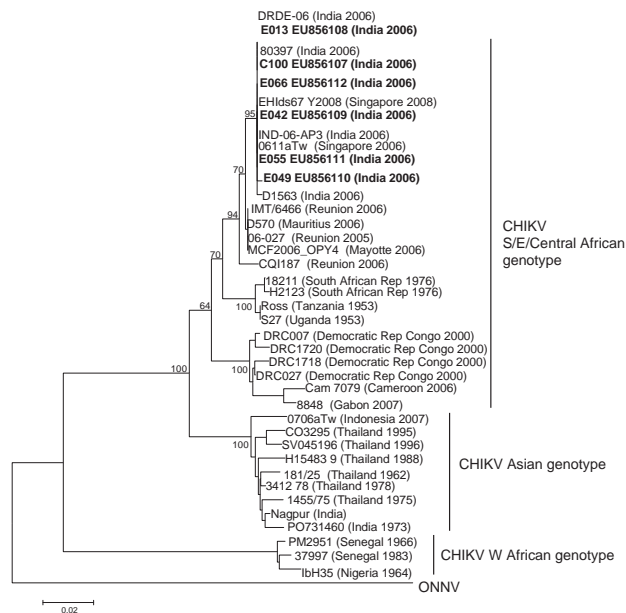
Figure 2. Phylogenetic analysis of chikungunya virus (CHIKV) sequences on the basis of partial E1 gene sequence (position 10620–11148 of the prototype CHIKV S27 genomic sequence). Sequences obtained in this study are in **boldface**. The analysis was performed using MEGA version 4 software (*8*), by using the neighbor-joining (p-distance) method. The length of the tree branches indicates the percentage of divergence; the percentage of successful bootstrap replicates is specified at the nodes (1,000 replicates). ONNV (o'nyong-nyong virus) prototype sequence was included to root the tree. Scale bar indicates number of nucleotide substitutions per site.

Acknowledgments

Dr Lewthwaite is an infectious diseases and tropical medicine physician who has undertaken her doctoral work in Japanese encephalitis at the University of Liverpool, UK. Her interests include neurologic infections, Japanese encephalitis, and emerging and imported infections.

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Effect of Free Treatment and Surveillance on HIV-Infected Persons Who Have Tuberculosis, Taiwan, 1993–2006

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In 1997, Taiwan made highly active antiretroviral therapy (HAART) available without cost to HIV-infected persons; in 2001, a national web-based surveillance system was implemented. Healthcare workers use the system to monitor patients' conditions and can intervene when necessary. Free HAART, coupled with the surveillance system, appears to have increased survival rates of HIV-infected persons with tuberculosis in Taiwan.

Most experts believe that complete and efficient surveillance is the top priority in detecting and preventing outbreaks of emerging infectious diseases, such as tuberculosis (TB) and HIV coinfection (1–3) or influenza virus A (H5N1). In Taiwan, a national web-based surveillance system established in July 2001 provides complete and efficient reporting and management of persons coinfected with HIV and TB and enables healthcare workers to identify noncompliance with therapy and to intervene when necessary. After highly active antiretroviral therapy (HAART) became available free of charge in Taiwan in April 1997, the death rate for HIV-infected persons decreased from 5.7% in 1997 to 1.8% in 2006. To determine whether implementation of the national surveillance system in combination with the availability of free HAART further increased survival rates of HIV-infected persons with TB, we compared their demographic, clinical, and behavioral characteristics during 3 periods: 1) before free HAART was available (1993–1996); 2) after free HAART was available but before the surveillance system was im-

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plemented (1998–2000); and 3) after both free HAART and the surveillance system were available (2002–2006).

The Study

We obtained data on persons with HIV/AIDS and TB from the national databank at the Centers for Disease Control (CDC Taiwan) of the Department of Health, Taiwan. Coinfection with HIV and TB was defined as HIV infection in persons in whom TB was later diagnosed. A total of 660 persons with both HIV and TB were reported during 1993–2006.

We used Microsoft Excel XP spreadsheet (Microsoft, Redmond, WA, USA) and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) for statistical analysis. The χ^2 goodness-of-fit test with type I error = 0.05 was used to examine differences in demographic, clinical, and behavioral characteristics of persons with HIV and TB coinfection during 1993–2006. Multivariates for analysis were sex and age, results of sputum smear and sputum culture, pulmonary radiographic diagnosis, TB types (extrapulmonary and nonextrapulmonary), mode of HIV transmission, sexual behavior, compliance with HAART, and use of the surveillance system (Table 1). We used the Kaplan-Meier method (4) from SAS to evaluate and compare the effect on survival rates of different factors in persons coinfected with HIV and TB 1 year after reported TB diagnosis.

Kaplan-Meier analysis yielded the following results: 63% of persons coinfected with HIV and TB survived during 1993–1996; 78% survived during 1998–2000; and 93% survived during 2002–2006 (p<0.0001) (Figure). We then applied Cox proportional hazards modeling (5) to each variable to assess the effect on survival after implementation of HAART and the surveillance system. Age ≤45 years, negative sputum smear, availability of free HAART, and implementation of the national surveillance system substantially increased survival rates of persons coinfected with HIV and TB (Table 2).

Conclusion

Many factors can increase survival rates of HIV-infected persons, such as HAART (6–9), prevention of opportunistic infections, patient attitude, healthcare worker knowledge, and promotion of health education. Our data indicate that national web-based surveillance reporting and management, coupled with the availability of free HAART, increase survival rates of persons coinfected with HIV and TB (p<0.0001).

Taiwan's national web-based surveillance system enables healthcare workers to follow, record, and understand the conditions of patients without geographic limitations. Physicians, public health nurses, health administrators, and other healthcare professionals in local through federal government agencies can use the system to follow up and man-

Table 1. Demographic and clinical characteristics of 660 persons coinfected with HIV and TB, Taiwan, 1993–2006*

Characteristics	No. (%) persons	p value†
Sex		<0.0001
M	612 (92.7)	
F	48 (7.3)	
Age, y		<0.0001
≤45	498 (75.5)	
>45	162 (24.5)	
Sputum smear (n = 484)		<0.0001
Negative	287 (59.3)	
Positive	197 (40.7)	
Sputum culture (n = 340)		0.0172
Negative	148 (43.5)	
Positive	192 (56.5)	
Pulmonary radiograph results (n = 531)		<0.0001
Normal	54 (10.2)	
Abnormal	477 (89.8)	
Extrapulmonary TB‡		<0.0001
Yes	73 (11.1)	
No	587 (88.9)	
Risk behavior (n = 554)		<0.0001
Sexual	513 (92.6)	
Injection drug user	41 (7.4)	
Sexual behavior (n = 566)		0.0004
Heterosexual	325 (57.4)	
Homosexual or bisexual	241 (42.6)	
Highly active antiretroviral therapy‡ (n = 534)		<0.0001
1998–2006 (free)	493 (92.3)	
1993–1996 (not free)	41 (7.7)	
National web-based surveillance reporting and management system (n = 520)		<0.0001
2002–2006 (available)	386 (74.2)	
1998–2000 (not available)	134 (25.8)	
Outcome§ (n = 606)		<0.0001
Survival	522 (86.1)	
Death	84 (13.9)	

*TB, tuberculosis.
†χ^2 goodness-of-fit test with type I error = 0.05 used to examine differences in demographic, clinical, and behavioral characteristics.
‡Definition following World Health Organization guideline (1).
§Tracking for 1 year from report of TB diagnosis.

age the condition of persons coinfected with HIV and TB. For example, public health nurses from national healthcare centers visit such patients regularly, record treatments, and assess their conditions and compliance with therapy; staff from central health department monitor and supervise the condition of each patient through the system. In this way,
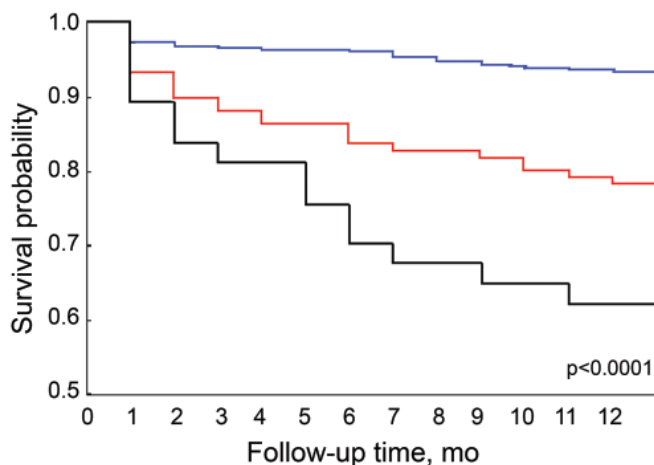

Figure. Kaplan-Meier analysis of survival of HIV-infected patients with tuberculosis in Taiwan during 3 different periods: before free highly active antiretroviral therapy (HAART) was available (1993–1996, black line); B) after free HAART was available but before the national web-based reporting and management surveillance system was implemented (1998–2000, red line); and C) after free HAART and the surveillance system were available (2002–2006, blue line).

Table 2. Cox regression model of possible risk factors for death among persons coinfected with HIV and tuberculosis, Taiwan, 1993–2006

Risk factor	Hazard ratio	95% Confidence interval	p value
Sex (F)	1.462	0.292–7.303	0.6438
Age (>45 y)	2.907	1.162–7.272	0.0226
Sputum smear positive	2.722	1.008–7.349	0.0482
Pulmonary radiograph abnormal	7.006	0.848–57.916	0.0708
Pulmonary tuberculosis	3.169	0.641–15.674	0.1573
Heterosexual	2.049	0.794–5.290	0.1333
Before availability of free highly active antiretroviral therapy*	8.398	2.170–32.508	0.0021
Before implementation of national web-based surveillance reporting and management system†	7.664	2.115–27.768	0.0019

*1993–1996.
†1998–2000.

the system may increase patients' compliance and thus their survival rates (*10–14*).

Acknowledgments

Dr Tseng is the deputy director of the Division of Surveillance and Nosocomial Infection Control at the Taiwan CDC. Her research interest focuses on coinfection of HIV and TB.

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Vaccine-induced Immunity Circumvented by Typical *Mycobacterium tuberculosis* Beijing Strains

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The frequency of typical and atypical Beijing strains of *Mycobacterium tuberculosis* was determined in the Netherlands; Vietnam; and Hong Kong Special Administrative Region, People's Republic of China. The strains' associations with drug resistance, *M. bovis* BCG vaccination, and patient characteristics were assessed. BCG vaccination may have positively selected the prevalent typical Beijing strains.

*M*ycobacterium tuberculosis Beijing strains cause a substantial proportion of tuberculosis (TB) cases worldwide (*1*). Experiments in a BALB/c mouse model (*2*) and a rabbit model (*3*) supported the hypothesis that Beijing strains might represent "escape variants" of *M. bovis* BCG vaccination (*4*). In a study in Ho Chi Minh City, Vietnam, presence of a BCG scar correlated, but not significantly, with infection by Beijing strains (*5*).

The Beijing clade is highly prevalent in Asia, where the proportion of TB cases caused by strains of this clade usually is stable over time, and no association with drug resistance has been recorded. In other areas (e.g., Cuba, South Africa, countries of the former Soviet Union, and Vietnam), Beijing strains are emerging and associated with resistance to anti-TB drugs (*1*). The Beijing clade comprises at least 2 major subgroups, which share the characteristic spoligotype pattern (*6–8*): typical and atypical Beijing

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strains. Typical ("modern" [*8,9*]) Beijing strains, including W strains (*7*), exhibit highly similar, multicopy insertion sequence (IS) *6110* restriction fragment length polymorphism (RFLP) patterns and have alterations in putative mutator genes (*4,10*). Atypical ("ancestral" [*8,9*]) Beijing strains more closely resemble the common ancestor of the Beijing clade (*6–8,10*). The ability of these Beijing clade subgroups to gain resistance or circumvent BCG vaccine–induced immunity may differ and thus explain the differences in geographic distribution of Beijing strains and the variation in association with drug resistance. However, few studies have distinguished between subgroups of the Beijing clade, or studies were limited in the number of strains analyzed (*8,9,11,12*).

The Study

We used 3 large data sets from previously described studies to investigate possible differences in correlation with resistance and BCG vaccination between sublineages of the Beijing clade. Details about drug-susceptibility testing, DNA fingerprinting, and demographics by origin can be found elsewhere (*5,13,14*). In the Netherlands, 415 (6%) of 6,829 *M. tuberculosis* isolates with available IS*6110* RFLP patterns from 1993 through 2000 were of the Beijing clade (*13*); approximately one third of cases each originated in the Netherlands and Asia and the remaining one third in other areas (*13*). In Vietnam, 301 (53%) of 563 isolates from new TB cases, collected during 1998–1999 mainly in Ho Chi Minh City, belonged to the Beijing clade (*5*). In Hong Kong Special Administrative Region, People's Republic of China, 355 (71%) of 500 randomly selected *M. tuberculosis* isolates collected during 1998–1999 from patients before treatment were of the Beijing clade (*14*). Information about patient sex and age was available from all 3 sites. Drug susceptibility data and BCG status of patients (presence/absence of BCG scar) were not available from Hong Kong. The patients in this study were treated according to World Health Organization guidelines, independent from their *M. tuberculosis* isolates' genotype.

Beijing clade strains were defined by their spoligotype pattern (*6*). We used the multiplex PCR of Plikaytis et al. (*15*) to differentiate 3 subgroups of the Beijing clade (W strain, typical, atypical). A specific IS*6110* insertion in the NTF region is detected in typical Beijing strains (*7*). W strains, a subgroup of typical Beijing strains, contain this IS*6110* and an additional IS*6110* insertion in this region (*7,15*). Figure 1 shows the correlation between the multiplex PCR results and IS*6110* RFLP similarity.

A total of 1,023 *M. tuberculosis* Beijing clade isolates (410 from the Netherlands, 268 from Vietnam, and 345 from Hong Kong) were available for multiplex PCR analysis. Mean age category of patients was 25–34 years, and >75% were <45 years of age. Because the W strain
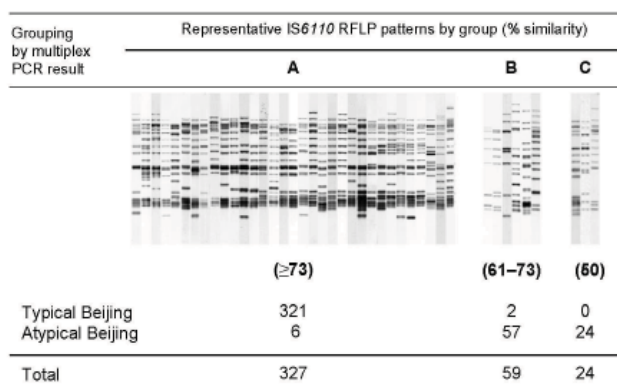
Grouping by multiplex PCR result	Representative IS*6110* RFLP patterns by group (% similarity)		
	A	B	C
	(≥73)	(61–73)	(50)
Typical Beijing	321	2	0
Atypical Beijing	6	57	24
Total	327	59	24

Figure 1. Correlation between the multiplex PCR results and insertion sequence (IS)*6110* restriction fragment length polymorphism (RFLP) pattern similarity. On the basis of IS*6110* RFLP pattern similarity, 3 groups of related patterns (A, B, and C) were recognized among 410 *Mycobacterium tuberculosis* Beijing clade strains isolated in the Netherlands. The similarity of the IS*6110* RFLP patterns of groups B (61%–73%) and C (50%) are relative to the RFLP patterns of group A. Within group C, the similarity of the patterns was at least 58%. By using an IS*6110* RFLP similarity of 73% as cutoff (which defined the most homogeneous IS*6110* RFLP group [group A]), 321 (98.2%) of 327 Beijing strains were consistently identified as typical Beijing, and 81 (97.6%) of the 83 remaining Beijing clade strains were classified as atypical Beijing by the multiplex PCR. The κ value for agreement between the tests is 0.922.

occurred infrequently, we included it in our analysis of the typical Beijing strain.

Typical and atypical subgroups were equally distributed among men and women but varied by country and patient age. Atypical Beijing strains occurred in 25.4% (68/268) of isolates in Vietnam, 21.2% (87/410) in the Netherlands, and 13.6% (47/345) in Hong Kong. Atypical Beijing strains were encountered less frequently in Hong Kong than in Vietnam (p<0.001) and the Netherlands (p = 0.007). The Beijing subgroups were equally prevalent among persons of different age groups in Hong Kong, but atypical Beijing strains occurred more frequently in older persons in the Netherlands and Vietnam (Figure 2). This increase in proportion of atypical Beijing strains in older persons was significant in the Netherlands (χ^2_{trend} 4.5, p<0.035). Combined data for the Netherlands and Vietnam showed significantly more atypical Beijing isolates among patients ≥75 years of age (odds ratio [OR] 2.96, 95% confidence interval [CI] 1.15–7.67) (Table 1), which suggests more recent introduction and spread of typical Beijing strains.

To determine whether BCG vaccination might drive this shift in prevalence of the 2 Beijing subgroups, we investigated their distribution in persons vaccinated and not vaccinated with BCG. Of 249 nonvaccinated persons, 27.7% were infected with atypical strains; of 265 vaccinated persons, a significantly lower proportion (20.8%) were infected with atypical strains (adjusted OR 0.60, 95%

CI 0.38–0.95) (Table 1). The proportions per genotype emphasized this finding; 44.4% of atypical Beijing strains and 53.8% of typical Beijing strains were isolated from vaccinated persons. The association between typical Beijing strains and vaccination was strong in the data from the Netherlands: 14.2% of Beijing strains isolated from vaccinated persons and 31% of those from nonvaccinated persons were atypical (adjusted OR 0.39, 95% CI 0.20–0.76). In Vietnam, the proportions were nonsignificant (26.2% and 24.4%, respectively).

The unknown BCG vaccination status of 164 of 678 Beijing strain–infected patients is a limitation of our study. In these patients (from the Netherlands), the proportion of atypical Beijing strains was lower and almost similar to that for vaccinated patients. Therefore, if all patients with unknown BCG status were considered nonvaccinated, the association with typical Beijing strains and BCG vaccination would disappear. However, the Netherlands' National Tuberculosis Register most likely lacks BCG status data because the BCG status for these patients was not checked; we assume the absence of these data introduced no bias. To investigate this further, we extended the analysis of the population in the Netherlands by including all patients with known BCG status (n = 4,004). The proportions of typical Beijing strains for BCG-vaccinated and BCG-nonvaccinated persons were 54.2% and 45.8%, respectively. For all strains other than Beijing strains (i.e., atypical Beijing strains and *M. tuberculosis* strains of all other genotypes), these proportions were 44.5% and 55.5%, respectively. Thus, including all isolates from persons with known BCG status, typical Beijing strains still were iso-
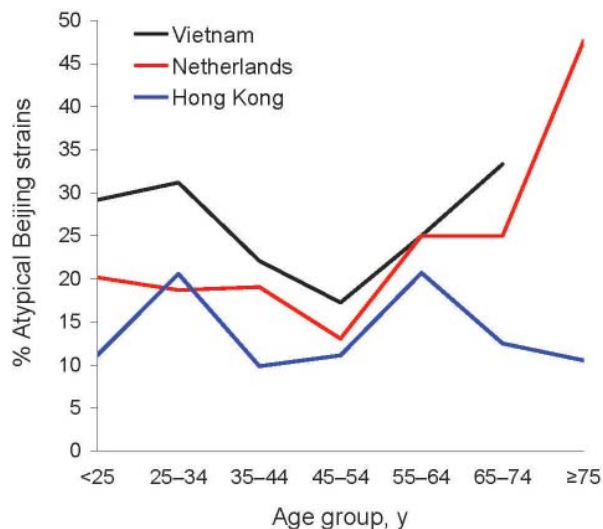

Figure 2. Proportion of atypical Beijing strains among persons with *Mycobacterium tuberculosis* Beijing clade strains in Vietnam, the Netherlands, and Hong Kong, by patient age. The data point of the ≥75-year age category from the data from Vietnam was omitted because the group contained only 1 patient.

Table 1. Risk factors for atypical *Mycobacterium tuberculosis* in 678 Beijing strain–infected persons with tuberculosis, Vietnam and the Netherlands

Risk factor	Total no. patients infected with Beijing clade	No. (%) patients infected with atypical Beijing strain	Odds ratio (95% confidence interval)	
			Crude	Adjusted*
Country				
Vietnam	268	68 (25.4)	1	1
The Netherlands	410	87 (21.2)	0.79 (0.55–1.14)	0.82 (0.52–1.29)
Sex				
M	415	93 (22.4)	1	1
F	219	48 (21.9)	0.97 (0.66–1.44)	1.01 (0.67–1.51)
Unknown	44	14 (31.8)	1.62 (0.82–3.17)	1.92 (0.90–4.11)
Age, y				
<25	164	36 (22.0)	1	1
25–34	215	51 (23.7)	1.11 (0.68–1.80)	1.05 (0.64–1.73)
35–44	140	29 (20.7)	0.93 (0.54–1.61)	0.80 (0.45–1.42)
45–54	51	7 (13.7)	0.57 (0.24–1.36)	0.45 (0.18–1.12)
55–64	36	9 (25.0)	1.19 (0.51–2.75)	1.03 (0.44–2.45)
65–74	50	13 (26.0)	1.25 (0.60–2.60)	1.07 (0.50–2.30)
≥75	22	10 (45.5)	2.96 (1.18–7.41)	2.96 (1.15–7.67)
BCG† vaccination				
No	249	69 (27.7)	1	1
Yes	265	55 (20.8)	0.68 (0.46–1.03)	0.60 (0.38–0.95)
Unknown	164	31 (18.9)	0.61 (0.38–0.98)	0.61 (0.36–1.04)
Total	678	155 (22.8)		

*Adjusted for country, sex, age group, and BCG vaccination.
†BCG, *Mycobacterium bovis* bacillus Calmette-Guérin.

lated significantly more often from BCG-vaccinated than BCG-nonvaccinated persons (p = 0.008). Our findings of significantly fewer typical Beijing isolates among patients ≥75 years of age (mostly experiencing reactivation and thus representing the population structure of *M. tuberculosis* of decades ago) and significantly more isolates of typical Beijing strains from BCG-vaccinated persons support the hypothesis that BCG vaccination might favor the spread of the typical Beijing strains.

Drug resistance of *M. tuberculosis* Beijing subgroups varied by country (Table 2). In Vietnam, drug resistance was significantly higher than in the Netherlands; 6.7% of Beijing strains in Vietnam compared with 2.0% in the Netherlands were multidrug resistant (MDR), 32.1% compared with 11.0% were isoniazid (INH) resistant, and 44.0% compared with 15.9% were streptomycin resistant (Table 2). Atypical Beijing isolates were more often INH resistant (25.2%) than were typical Beijing isolates (17.6%). Furthermore, atypical Beijing strains were more often MDR (7.1%, compared with 2.9%). Atypical Beijing strains were less often streptomycin resistant (21.3% compared with 28.7%) (Table 2). Thus, atypical Beijing isolates were associated with INH resistance and MDR and significantly less likely to be streptomycin resistant than typical Beijing isolates. Similar differences in drug resistance recently were found for the 2 Beijing subgroups among isolates circulating in Japan (*12*) and in the Beijing region of China (*9*), but the findings in China were not statistically significant, probably because of the limited number of stains analyzed. These differences in drug resistance associations suggest the different Beijing sublin-

eages might have different mechanisms of drug resistance development.

Despite the association of atypical Beijing strains with INH and multidrug resistance found in this study, typical Beijing strains contribute most substantially to the worldwide MDR TB epidemic (*1,4,11*). However, in studies showing an association between typical Beijing strains and multidrug resistance, these strains usually also were resistant to streptomycin (as we also found). Typical Beijing strains may therefore become streptomycin resistant more easily, eventually leading to MDR TB, as the W-strain outbreak in New York showed (*16*). Alternatively, the increased prevalence of typical Beijing strains in the current global *M. tuberculosis* population may be caused not by drug-driven selection but by their hypervirulence (*2*), higher adaptability (*10*), higher rate of progression to disease, greater ability to circumvent BCG-induced immunity (*2,3*, this study), or other specific features.

Conclusions

We showed that subgroups of the *M. tuberculosis* Beijing clade have different associations with drug resistance and BCG vaccination. Individual lineages of the Beijing clade are likely to be evolving in different areas, possibly because of intrinsic strain characteristics, differences in anti-TB drug regimens and BCG-vaccination strategies in different areas, chance, or a combination of these. Thus, anti-TB drugs and BCG vaccination influence the dynamics in the population structure of *M. tuberculosis*. The efficacy of new candidate TB vaccines therefore should be tested against a broad panel of epidemic strains from

Table 2. Risk factors for drug resistance of *Mycobacterium tuberculosis* Beijing clade strains in 678 persons with tuberculosis, Vietnam and the Netherlands*

Risk factor	Total no. patients	Resistance, % patients			Adjusted odds ratio (95% confidence interval)†		
		Multidrug	INH	SM	Multidrug	INH	SM
Genotype							
Typical Beijing	523	2.9	17.6	28.7	1	1	1
Atypical Beijing	155	7.1	25.5	21.3	2.48 (1.09-5.65)	1.58 (1.00–2.49)	0.59 (0.37–0.93)
Country							
Vietnam	268	6.7	32.1	44.0	1	1	1
The Netherlands	410	2.0	11.0	15.9	0.21 (0.06–0.76)	0.20 (0.11–0.34)	0.21 (0.13–0.33)
Sex							
M	415	3.6	18.6	28.0	1	1	1
F	219	2.7	19.2	22.4	0.91 (0.34–2.44)	1.34 (0.86–2.11)	0.87 (0.58–1.31)
Unknown	44	11.4	27.3	40.9	2.39 (0.67–8.54)	0.90 (0.41–1.97)	0.88 (0.43–1.78)
Age, y							
<25	164	3.7	13.4	25.0	1	1	1
25–34	215	4.7	24.7	28.4	0.93 (0.32–2.70)	1.76 (1.00–3.12)	0.94 (0.57–1.54)
35–44	140	4.3	25.0	30.0	0.71 (0.21–2.41)	1.46 (0.78–2.74)	0.82 (0.47–1.43)
45–54	51	3.9	17.6	33.3	0.68 (0.12–3.77)	0.91 (0.37–2.27)	0.92 (0.44–1.95)
55–64	36	2.8	16.7	27.8	0.60 (0.07–5.45)	0.99 (0.35–2.81)	1.05 (0.44–2.52)
65–74	50	2.0	10.0	16.0	0.40 (0.04–3.66)	0.63. (022–1.85)	0.57 (0.23–1.39)
≥75	22	0	4.5	18.2	--	0.31 (0.04–2.50)	1.06 (0.32–3.49)
BCG vaccination							
No	249	4.0	22.1	28.9	1	1	1
Yes	265	4.2	19.6	30.9	0.63 (0.22–1.83)	0.71 (0.43–1.17)	0.99 (0.63–1.54)
Unknown	164	3.0	14.6	17.7	2.44 (0.54–11.04)	1.64 (0.84–3.21)	1.26 (0.70–2.24)
Total	678	26	131	183			

*INH, isoniazid; SM, streptomycin; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin.
†Adjusted for genotype, country, sex, age group, and BCG vaccination.

all high-prevalence areas (*4*). Furthermore, treatment of infections by different *M. tuberculosis* genotypes might require different anti-TB treatment strategies. More extended studies are needed in high-prevalence settings, especially studies of other predominant genotype families of *M. tuberculosis*.

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Serogroup W135 Meningococcal Meningitis, Northern Cameroon, 2007–2008

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We analyzed results of recent microbiologic surveillance of meningitis in northern Cameroon. During the 2007 and 2008 meningitis seasons, all 57 identified meningococcal isolates were serogroup W135. This situation might indicate that the area is experiencing a period between epidemic waves due to 2 different clones of serogroup A meningococci.

The 3 provinces constituting the Septentrion, in North Cameroon, belong to the so-called African meningitis belt (*1*). Until now, in these provinces, the diagnosis of meningitis was made essentially on the basis of clinical signs, and biologic confirmation was uncommon. The rare data that documented meningococci circulating in recent years in this area refer mostly to the early 1990s (*2,3*), when serogroup A was by far the most frequently identified serogroup in Cameroon, with strains having the antigenic formula A:4:P1.9 and belonging to the sequence type 5 (ST-5) complex (*4*). From 1999 through 2001, a few cases of meningitis were attributed to serogroup W135 (W135:2a:P1.5,2; ST-11), but these data were from Yaounde, in the southern part of the country (*5*).

The Study

In 2007, microbiologic surveillance of bacterial meningitis was reintroduced in northern Cameroon to monitor the changing epidemiology of meningococci, with particular attention to uncommon serogroups, such as W135 or X, which have been unusually frequent in Sahelian Africa since 2000 (*6–10*). Health authorities decided that an aliquot of every cerebrospinal fluid (CSF) specimen collected in this area would be sent to the Centre Pasteur du Cameroun in Garoua (CPCAG) for testing. Laboratory proce-

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dures included assessment of CSF turbidity, Gram staining, search for soluble capsular antigens by using the Pastorex (Bio-Rad, Hercules, CA, USA) latex agglutination kit and dipstick rapid diagnostic test for *Neisseria meningitidis* serogroups A, C, W135, and Y (kindly provided by the Centre de Recherche Médicale et Sanitaire, Niamey, Niger) (*11*). CSF specimens were cultured on blood agar and chocolate agar, supplemented with Polyvitex (bioMérieux, Marcy-l'Etoile, France), with incubation at 37°C with 5% CO_2. Susceptibility to β-lactam antimicrobial drugs and chloramphenicol was tested according to the recommendations of the Antibiogram Committee of the French Society for Microbiology. The isolates of *N. meningitidis* were sent to the World Health Organization Collaborating Centre (WHOCC) for Reference and Research on Meningococci in Marseilles, France.

Overall, 409 CSF specimens were tested at CPCAG microbiology laboratory from January 1, 2007, through June 30, 2008, of which 144 (35.2%) had a leukocyte count evocative of bacterial meningitis. Online Appendix Figure 1 (available from www.cdc.gov/EID/content/15/2/340-appF1.htm) shows the monthly distribution of CSF specimens tested at CPCAG.

The number of CSF specimens tested at CPCAG increased greatly after 2006 as the number of health facilities sending specimens for testing increased (Table). This rise was not related to any epidemic, but showed the interest of healthcare workers in microbiologic surveillance of meningitis. The number of tested CSF specimens peaked in March and April, during the dry season, and was low during the rainy season (August and September). Overall, 24 *Streptococcus pneumoniae*, 23 *Haemophilus influenzae*, and 57 *N. meningitidis* isolates were identified, either from culture or by soluble antigens identification.

S. pneumoniae and *H. influenzae* were identified throughout the year, but *N. meningitidis* was observed only during the dry season, considered the classical meningitis season in the African meningitis belt (*1*). The median ages of 11 patients with confirmed *S. pneumoniae* infection and 11 patients with confirmed *H. influenzae* infection were 14 and 0.6 years, respectively. Neither *S. pneumoniae* (*12*) nor *H. influenzae* isolates (*8*) were serotyped. The median age of 43 patients with confirmed meningococcal meningitis was 8 years (25th percentile 4.8 years, 75th percentile 15.5 years).

Fifteen *N. meningitidis* isolates were recovered from culture, representing 28.3% of CSF specimens testing positive for soluble antigens. The frequent implementation of early presumptive treatment with antimicrobial drugs, as well as the unsuitable conditions for CSF specimens transport from health facilities, might account for this low rate of positive cultures. The implementation of PCR for the diagnosis of *N. meningitidis*, *S. pneumoniae*,

Table. CSF specimens from Cameroon tested at CPCAG laboratory in 2007 and 2008 and number of health facilities that sent specimens, compared with 2006 efforts*

Characteristic	2006	2007	2008†
No. tested CSF specimens	60	202	207
Health facilities involved	Garoua Hospital	Garoua Hospital plus 8 other health facilities	Garoua Hospital plus 17 other health facilities
Specimens not collected in Garoua	0	27	79

*CSF, cerebrospinal fluid; CPCAG, Centre Pasteur du Cameroun in Garoua.
†Through June 30.

and *H. influenzae* in CPCAG in Garoua in 2009 will enable further enhancement of the surveillance.

All diagnosed meningococci belonged to serogroup W135, although serogroup A has been classically the most prevalent in this Sahelian region, according to surveys carried out in Garoua (*2,3*). *N. meningitidis* serogroup A seems to have completely disappeared since the renewal of the microbiologic surveillance of meningitis in northern Cameroon began in February 2007. To our knowledge, there was no previous report in the literature of such a predominance of serogroup W135 in countries of the meningitis belt.

Nine of our 15 *N. meningitidis* isolates were genotyped by using multilocus sequence typing by the WHOCC in Marseille. Eight were identified as ST-2881 and 1 as ST-11. These findings are consistent with the presence of only sporadic cases of serogroup W135 meningococcal meningitis. Until now, ST-2881 has only been associated with sporadic cases or small epidemics, contrary to the findings for ST-11, which was involved in a large epidemic in Burkina Faso in 2002 (*10,12*).

We did not observe any resistance of *N. meningitidis* to β-lactam antimicrobial agents and chloramphenicol. These findings did not question probabilistic treatment of suspected cases of meningococcal meningitis based on ceftriaxone or oily chloramphenicol in epidemic situations (*13*).

Laboratory-confirmed cases of serogroup W135 meningococcal meningitis were from Garoua and 9 other health facilities (online Appendix Figure 2, available from www.cdc.gov/EID/content/15/2/340-appF2.htm). Most cases were from the North and Extreme North Provinces. Adamaoua Province seemed to be less affected, likely because of climate and lower population density.

Our results differ clearly from those observed in Yaounde, the capital, which has a subequatorial climate. In 1999–2000, *S. pneumoniae* was the main causative bacterial agent identified in meningitis (109/194, 56%), followed by *H. influenzae* (36/194, 18.6%) and *N. meningitidis* (26/194, 13.4%) (*14*).

WHO does not recommend systematic preventive vaccination with the bivalent A + C polysaccharide vaccine, but does recommend reactive mass immunizations of at-risk populations when the epidemic threshold is crossed. This policy was applied in northern Cameroon in 1992, 1993, 1996, and 1998, when >400,000 doses of vaccine were administered each year for epidemic control. After

this period, 30,000–40,000 persons received preventive immunization against *N. meningitidis* serogroups A and C each year. However, the vaccination coverage is too low in this population of ≈6 million inhabitants to explain by itself the current absence of serogroup A meningococci in this area.

Conclusions

The renewal of microbiologic surveillance of meningitis highlighted the fact that serogroup W135 was the only serogroup of *N. meningitidis* involved in endemic meningitis cases in northern Cameroon in 2007 and 2008. Serogroup A meningococci seemed to be completely absent during this period. This finding might reflect the transitory period separating 2 epidemic waves caused by 2 different clones of serogroup A meningococci (*15*); the emergence of the next epidemic clone is thus possible at any moment. In the meantime, this situation has been considered by WHO, and vaccines that include protection against serogroup W135, such as trivalent vaccine (ACW135), are available for immunization campaigns to control epidemics. This vaccine is available in small quantities and can be delivered only by the International Coordinating Group (created in 1997 by WHO to distribute vaccine ACW135). WHO does not recommend this vaccine when meningococcal meningitis is endemic. Unless an epidemiologic upheaval occurs, future large epidemics of meningococcal meningitis will likely be caused by serogroup A, and the serogroup A conjugate vaccine (which will soon be introduced in the meningitis belt) will be a major advance. However, its successful implementation might create a favorable climate for other serogroups such as W135. In the future, a low-cost conjugate vaccine that includesW135 and perhaps X and Y serogroups can be anticipated.

In the early 2000s, scientists and WHO expressed fear of epidemics caused by serogroup W135 in countries of the African meningitis belt, but the epidemic in Burkina Faso remained isolated. Because the W135 serogroup ST-2881 is known to be less epidemic than W135 ST-11, ST-2881 could have replaced it in the ecologic niche and also have resulted in immunologic protection against ST-11. The observations made in northern Cameroon confirm that we must continue to take into account serogroup W135 and that laboratories should be able to identify it routinely.

Acknowledgments

Dr Massenet is a medical biologist who directs the annex of the Centre Pasteur du Cameroun in Garoua. His research interests include strengthening laboratory capacities in sub-Saharan Africa toward a better control of infectious diseases such as bacterial meningitis.

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Clostridium difficile–associated Disease in the Elderly, United States

To the Editor: Zilberger et al. (*1*) recently commented on the increase of hospitalizations for *Clostridium difficile*–associated disease (CDAD) and noted an increase in the case-fatality rate during 2000–2005. These findings refer to the entire US adult population and agree with our observations for the elderly (≥65 years of age). We assessed trends of CDAD in the elderly by using hospital billing data from the Centers for Medicare and Medicaid Services (CMS), which covers 98% of the elderly population (*2*). We abstracted all 1,054,125 hospitalization records that included *C. difficile* (International Classification of Diseases, 9th revision, Clinical Modification [ICD 9-CM], diagnosis code 008.45) in any of the 10 diagnosis code positions for a 14-year period (1991–2004). We used elderly-population data from the 1990 and 2000 US Census. The ICD code for *C. difficile* was introduced in 1992. Case-patients in our dataset prior to this date represent severe illness and were hospitalized for >1 year and therefore were still in the hospital when the ICD code was introduced. We considered data from 1993 through 2004 because 1991 and 1992 are not representative due to introduction of the ICD code.

We observed an increase in overall hospitalizations that included a diagnosis for CDAD (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/15/2/343-appF.htm) and an increase in rates of CDAD from 13.71/10,000 elderly in 1993 to 38.78/10,000 in 2004 (*3*). The highest rate of hospitalizations was detected in the oldest patients (≥85 years of age), 48.2/10,000 vs. 11.9 in those 65–74 years of age and 26.0 in those 75–84 years of age (*3*). These rates might be higher than rates reported by Zilberberg et al. because our records account for all treated conditions recorded by all 10 diagnosis codes. The ICD code for CDAD typically does not appear in the primary and secondary diagnosis; overall, 60% of all CMS records list CDAD as codes 3–10 (*3*). Primary and secondary codes typically represent diagnoses for which the patient is admitted, whereas diagnosis codes 3–10 are codes used for chronic conditions and sequelae. The online Appendix Figure, panel A, shows the change in the proportion of CDAD cases in each diagnosis code over the study period. The proportion of CDAD in the primary and secondary diagnosis position increased during 1996–1997; however, this proportion is stabilizing at ≈25%.

Zilberberg et al. observed a doubling in age-adjusted case-fatality rates from 1.2% in 2000 to 2.2% in 2004 (*1*), which is an annual increase of 0.2% over the 5-year period. We are not able to calculate case-fatality rate by using CMS data because these data do not provide cause of death, only an indicator of whether the patient died during that hospital stay. However, we observed an increase in the percentage of patients with CDAD who died, from 8.8% in 1993 to 9.7% in 2004, which is an annual increase of 0.075% over the 12-year period. We also observed a peak in 2000; 10.4% of patients with CDAD died. This peak is unusual and unexplained and requires further analysis. Data on deaths must be interpreted with caution because they may be affected by severe conditions and age (oldest patients).

We observed an increasing trend and strong seasonal pattern in CDAD hospitalizations. The online Appendix Figure, panel B, shows this seasonal pattern by week during 1993–2004. This figure shows an increasing trend over time with a sharp change in slope in 2001. This increasing trend may represent an increase in disease or may be caused by increased testing and recognition of disease. Diagnosis of CDAD in the United States is now made by using an enzyme immunoassay that is relatively easier and cheaper to perform than a cytotoxin assay (*4*), which may account for the increased trend.

Increases in rates of CDAD may be caused by a reporting bias of gastroenteric diseases (*5–7*). To assess this possibility, we extracted all records that included other infectious gastroenteritis without CDAD (all other gastrointestinal [GI] infections, ICD 001–009 without 008.45) and compared the trend with CDAD hospitalizations (online Appendix Figure, panel B). The online Appendix Figure shows that rates for all other GI infections remained fairly constant over the study period, and a reporting bias for GI infections does not account for the ≈3-fold increase in CDAD hospitalizations. CDAD hospitalization rates for the elderly also show a strong annual seasonal pattern (online Appendix Figure, panel B), which was estimated to peak in the second week of March, the 10th week of the year. This seasonality suggests dominant routes of transmission that may be environmentally driven.

Our findings support the observations of Zilberberg et al. and demonstrate the substantial increase in CDAD-related hospitalizations over time. These findings and the aging population in the United States underscore the need for further research to understand all aspects of CDAD.

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Viral Etiology of Common Cold in Children, Finland

To the Editor: The common cold is regarded as a viral disease. In the first years of the 21st century, several new respiratory viruses have been identified, such as human metapneumovirus (hMPV), coronaviruses NL63 and HKU1, and human bocavirus (HBoV). Many studies have addressed the role of these viruses in hospital settings, but few studies have been conducted among outpatients. We examined the etiology of the common cold in young children who were newly symptomatic but had no need of hospital care. We hypothesized that the etiology could be detected in all cases by using modern diagnostics that test for 16 viruses in outpatients.

Between February 1996 and April 1998, we collected nasopharyngeal aspirate samples in an outpatient setting from 194 Finnish children having newly onset (<48 h) symptoms of common cold but no acute otitis media (AOM) or other symptoms demanding antimicrobial therapy (*1*). The mean age of the study population was 2.1 years (range 0.7–3.9 years), and 81% attended day care. The parents of all participants gave written informed consent, and the study protocol was approved by the Ethics Committee of Turku University Hospital in Turku, Finland.

The nasopharyngeal aspirate samples were processed freshly for antigen detection (respiratory syncytial virus [RSV]; parainfluenza viruses 1, 2, and 3; influenza A and B viruses; and adenovirus) by time-resolved fluoroimmunoassay (*2*). Stored samples were subjected to nucleic acid testing (NAT) for picornaviruses; RSV; coronaviruses 229E, OC43, NL63, and HKU1; influenza C virus; HBoV; hMPV; and adenovirus. Recently, these samples were reanalyzed for rhinovirus and enterovirus using real-time PCR for the amplification step (*1,3–6*).

At least 1 respiratory virus was detected in 179 (92%) of 194 children. Rhinovirus was the most common respiratory virus, found in 138 (71%) children (Table). Other viruses were found in varying proportions: HBoV was present in 27 (14%) children; adenovirus was found in 23 (12%) (3 were positive by antigen detection, and 23 by NAT); enterovirus was present in 20 (10%); coronaviruses were found in 11 (6%) (NL63:7; HKU1:2; 229E/OC43:2); influenza viruses were present in 11 (6%) (A:4; B:1; C:6); RSV was shown in 8 (4%) (all were positive by antigen detection and NAT); parainfluenza viruses were present in 7 (4%) (1:1; 3:6); and hMPV was found in 3 (2%). The Table shows the concomitant occurrence of all viruses. Among children with a positive viral finding, 46 (26%) had 2 viruses, and 10 (6%) had 3 or 4 viruses concomitantly. The viruses occurring most frequently with other viruses were adenovirus (100%), HBoV (81%), and enterovirus (75%).

Although our diagnostic panel was incomplete, lacking parechoviruses and parainfluenza type 4 virus, we detected ≥1 respiratory viruses in 92% of the children who had a common cold. As expected, rhinovirus was the leading cause of the common cold in these children. The role of picornaviruses was also emphasized by the abundance of enteroviruses. Enterovirus has gained attention mainly in severe infections, e.g., meningoencephalitis, and is rarely included in diagnostics for respiratory infections. However, PCR has shown that enterovirus commonly causes upper and lower respiratory infections that may be complicated by AOM or expiratory wheezing (*4,7*). Thus, enterovirus should be included in the diagnostic panels of respiratory infections. HBoV was the second most prevalent virus in our study population. Since its discovery in 2005, HBoV positivity has been

reported in 3%–19% of different study populations (*8*). Its pathogenic role has been questioned because most HBoV cases are co-infections with other viruses (*8*), and 81% of those testing positive for HBoV in our study had co-infections. However, adenovirus and enterovirus reached similar co-infection frequencies, likely because of prolonged postinfection viral shedding of these agents. HBoV–specific immunoglobulin (Ig) M and IgG antibody responses were recently reported in children with wheezing, suggesting that HBoV induces a systemic infection and is probably a true causative agent of lower respiratory tract disease

(*9*). Our study indicates that HBoV may also be a common cause of common cold in young children. However, we found hMPV, coronaviruses NL63 and HKU1, and influenza C virus in 1%–4% of the children, suggesting that these viruses play a minor role in childhood common cold. Our study may underestimate the role of RSV and hMPV because we excluded children with AOM, which is frequently related to these viruses.

Multiple viral findings were common in our study, and 3 children had 4 viruses concomitantly, a logical finding because young children are constantly exposed to respiratory viruses,

especially if they attend day care. A recent follow-up study showed that almost all viral findings were related to symptoms, thus supporting the argument that most, if not all, viruses are causative agents (*10*).

A possible causative agent of the common cold can be found in nearly all children who have a cold, and rhinovirus is the leading causative agent. In our study, HBoV was also found frequently, but the recently discovered viruses hMPV and coronaviruses NL63 and HKU1 played a minor role in the common cold of young children.

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Table. Positive viral findings in 194 children with newly onset uncomplicated common cold, Finland, 1996–1998

Virus	No. (%) positive*
Rhinovirus	91 (47)
Rhinovirus and human bocavirus	13 (7)
Rhinovirus and adenovirus	11 (6)
Rhinovirus and enterovirus	6 (3)
Human bocavirus	5 (3)
Enterovirus	5 (3)
Respiratory syncytial virus	5 (3)
Influenza C virus	4 (2)
Parainfluenza virus 3	4 (2)
Rhinovirus, adenovirus, and enterovirus	3 (2)
Coronavirus NL63	2 (1)
Human metapneumovirus	2 (1)
Coronavirus 229E or OC43	2 (1)
Rhinovirus and parainfluenza virus 3	2 (1)
Rhinovirus and influenza A virus	2 (1)
Human bocavirus and enterovirus	2 (1)
Adenovirus and enterovirus	2 (1)
Rhinovirus, adenovirus, and coronavirus NL63	2 (1)
Rhinovirus, human bocavirus, adenovirus, and enterovirus	2 (1)
Influenza A virus	1 (1)
Influenza B virus	1 (1)
Coronavirus HKU1	1 (1)
Rhinovirus and respiratory syncytial virus	1 (1)
Rhinovirus and coronavirus NL63	1 (1)
Rhinovirus and parainfluenza virus 1	1 (1)
Human bocavirus and respiratory syncytial virus	1 (1)
Human bocavirus and coronavirus NL63	1 (1)
Human bocavirus and influenza C virus	1 (1)
Adenovirus and respiratory syncytial virus	1 (1)
Coronavirus NL63 and influenza A virus	1 (1)
Rhinovirus, human bocavirus, and influenza C virus	1 (1)
Rhinovirus, adenovirus, and human metapneumovirus	1 (1)
Rhinovirus, human bocavirus, adenovirus, and coronavirus HKU1	1 (1)
Total positive	179 (92)
Total negative	15 (8)
Total children sampled	194 (100)

*Percentages rounded to nearest whole number.

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Time from Illness Onset to Death, 1918 Influenza and Pneumococcal Pneumonia

To the Editor: Brundage and Shanks (*1*) have studied time to death from the onset of influenza symptoms during the 1918 pandemic in military and civilian populations and found a median time to death of 7–11 days. They argue that these data support the idea that the deaths may be predominantly due to bacterial superinfection after the acute phase of influenza. We observed a similar 10-day median time to death among soldiers dying of influenza in 1918 (*2*), a finding consistent with the time to death for a bacterial superinfection, specifically pneumococcal bacteremic pneumonia (*3*).

The major bacterial pathogen associated with influenza-related pneumonia in 1918 was *Streptococcus pneumoniae* (*1*,*3*). Neither antimicrobial drugs nor serum therapy was available for treatment in 1918.

To further analyze the time course of death from influenza in relation to that of pneumococcal pneumonia in 1918, we examined data collected by Tilghman and Finland (*4*) from the pre–antimicrobial drug era of the 1920s and 1930s. The Figure shows the distribution of time from onset of illness to death due to influenza-related pneumonia in 1918 compared with time to death due to untreated pneumococcal pneumonia in the 1920s and 1930s. The Figure indicates a close concordance of the times to death. Similar times to death do not prove the specific bacterial etiology of the 1918 deaths. However, pneumococcal bacteremia was associated with most of the pneumonia deaths reported by Tilghman and Finland (*4*), and most 1918 influenza-related deaths were due to bacterial pneumonia (*5*). Also, up to 50% of patients dying from pneumonia in 1918 had pneumococcal bacteremia (*3*). These similar times to death provide additional evidence that the influenza-related pneumonia deaths during the 1918 influenza pandemic were largely due to the pneumococcus.
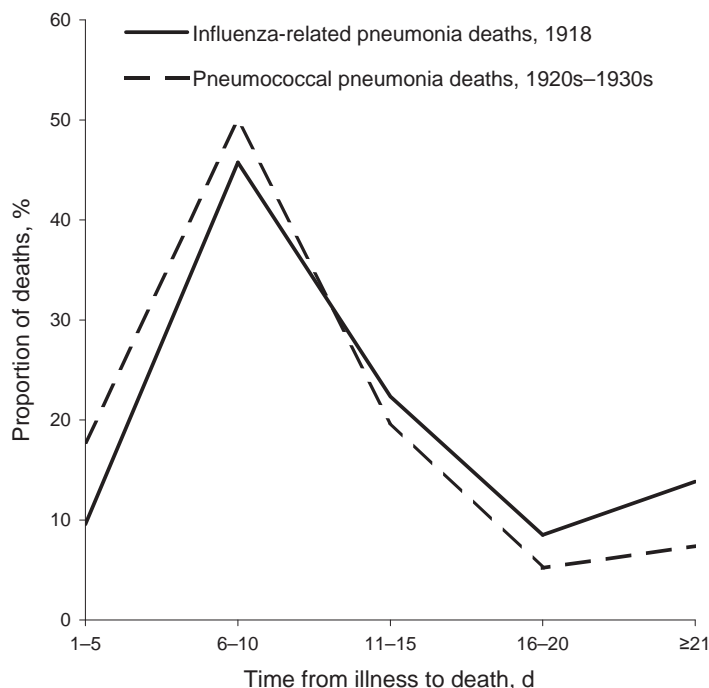

Figure. Distribution of days of illness before death from influenza-related pneumonia, 1918, and from untreated pneumococcal pneumonia, 1920s and 1930s.

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Unusual Manifestation of Toscana Virus Infection, Spain

To the Editor: Toscana virus (TOSV) causes acute meningitis and meningoencephalitis in Mediterranean countries (*1*). In Spain, neurologic TOSV infection has been reported since 1988. All cases have been self-limited aseptic meningitis (*2*). Since 2003, we have routinely investigated TOSV in cerebrospinal fluid (CSF) specimens from patients with suspected viral meningitis and encephalitis by using cell culture and reverse transcription–PCR (RT-PCR) (*3,4*). Also, as part of a regional project (05/305, Consejería de Salud, Junta de Andalucía, Spain), we investigated TOSV in mild nonneurologic syndromes by detection of immunoglobulin (Ig) M against TOSV by using enzyme immunoassay (Diesse Diagnostica Senese S.p.A, Siena, Italy). From May through September of 2006 and 2007, a total of 358 serum samples were randomly selected from patients for whom microbiologic determinations had been requested to investigate febrile illnesses.

As a result of these virologic and serologic surveys, we detected 7 cases of TOSV infection. Mild aseptic meningitis developed in 4 patients; in 3 patients, the infection had an atypical manifestation, as described below.

Patient 1, a 45-year-old man, was referred to the Hospital Universitario Virgen de las Nieves in September 2004 with confusion and a temperature of 39°C. He had had a splenectomy 20 years before, and in 2002, he had received a kidney transplant after renal failure resulting from meningococcemia. On admission, the patient was receiving chronic immunosuppressive treatment. Ten days after admission, he had tonic-clonic seizures. Aphasia and paresis developed after an ictus of the left hemisphere, and his level of con-

sciousness decreased rapidly. Treatment with corticosteroids was initiated because vasculitis was suspected. The patient responded to treatment, and 2 months after admission, he was discharged. Four months later, he still had impaired speech and paresis. Lymphocytic pleocytosis, a normal glucose level, and elevated protein levels were observed in CSF samples taken during the 2-month period of hospitalization. Bacterial and fungal cultures, as well as results of PCR for enterovirus, herpes simplex virus (HSV), and varicella-zoster virus (VZV), were negative in CSF specimens taken at admission and 1 month later. TOSV was detected by cell culture and nested RT-PCR in both samples (*3*). Anti-TOSV IgG was not detected in serum samples obtained on days 1 and 10; 5 months later, a borderline result was obtained. Anti-TOSV IgM was not detected on day 1 but was detected on day 10; 5 months later, anti-TOSV IgM was detected. Sequence analysis of amplified fragments from L and S segments (GenBank accession nos. FJ356705 and FJ356706, respectively) indicated 95%–98% homology with sequences from Spanish TOSV strains (*3*) and 84% homology with Italian reference strain ISS Phl.3.

Patient 2, a 54-year-old man, was admitted to a regional hospital in Granada Province in November 2007. He was confused and agitated, and he reported having fever and headache 2 days before. On admission, he was receiving treatment with corticosteroids for Crohn disease. Analysis of the CSF specimen showed lymphocytic pleocytosis, a normal glucose level, and increased protein levels. Results of PCR for HSV, VZV, and enterovirus were negative. TOSV was detected in the CSF sample by cell culture and real-time RT-PCR (*4*). The patient was treated with antimicrobial drugs and acyclovir. He recovered and was discharged 3 weeks after admission. One month later, he returned with paresis and aphasia, secondary to an ischemic

stroke located in the left hemisphere. IgG and IgM antibodies against TOSV were detected in a serum sample obtained at that time. The patient was discharged 1 week later with slight aphasia.

The most relevant common signs observed in patients 1 and 2 were the ischemic complications. Few cases of complicated encephalitis with sequelae caused by TOSV have been described (*5,6*). Moreover, to our knowledge, persistent neurologic TOSV infection has not been reported. The immune status of these patients probably influenced the clinical outcome in both patients and the delayed serologic response in patient 1.

Patient 3, a 41-year-old woman, sought treatment for exanthema at her health care center in July 2004. Test results for IgM antibodies against rubella, parvovirus B19, and *Rickettsia conorii* were negative. Specific anti-TOSV IgM was detected. The infection was self-limited, and no signs of neurologic involvement were associated with the rash. This was the only case of anti-TOSV IgM detection in 358 serum specimens analyzed from patients with nonneurologic syndromes. Although this finding is not conclusive, it suggests that TOSV infection might be involved occasionally in other mild syndromes. Two other cases of TOSV infection without neurologic involvement have been reported elsewhere: febrile erythema in Italy (*7*) and an influenza-like illness in southern France (*8*).

The unusual manifestations of TOSV infection reported here occurred in persons from rural areas within Granada Province, where seroprevalence rates have been shown to be higher than in urban areas (*3*). These data provide more information about this arboviral infection. Atypical TOSV infection could occur particularly in areas where the virus is endemic.

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Sporadic Oropouche Virus Infection, Acre, Brazil

To the Editor: *Oropouche virus* (OROV), a member of the *Bunyaviridae* family, *Orthobunyavirus* genus, Simbu serogroup, is transmitted to humans in urban areas by the biting midge *Culicoides paraensis* and causes epidemic acute febrile disease (*1*). Since its first isolation in Trinidad in 1955 (*2*), OROV has been associated with large outbreaks in South and Central America; half a million cases have been described during the past 45 years (*1*). The tripartite genome of OROV comprises single-strand, negative-sense large (L), medium (M), and small (S) RNAs that encode RNA polymerase, glycoproteins, and nucleocapsid, respectively. Studies have indicated the existence of 3 genotypes of OROV circulating in Brazil: genotypes I and II in the Amazon Basin and genotype III in the Southeast Region (*3–5*).

OROV causes explosive urban epidemics. Serologic evidence of exposure to OROV in populations not affected by known outbreaks suggests that the virus circulates endemically (*1*). However, no sporadic infections have been reported. Here we report a sporadic OROV infection detected by clinical and laboratory surveillance of acute febrile illnesses in Acre, a state in the western Amazon region of Brazil. From March 2004 through October 2006, we prospectively investigat-

ed 69 febrile episodes in persons 6–60 years of age (mean, 28.1 years) living in the town of Acrelândia (10°13′W, 67°00′S) and surrounding rural areas (25.7% and 74.3% of the sample, respectively).

Serum samples for reverse transcription–PCR (RT-PCR) were stored in liquid nitrogen in the field and shipped on dry ice to the laboratory in São José do Rio Preto, 3,500 km southeast of Acre. Because malaria and several arboviruses are locally endemic (6), all patients were screened for malarial parasites by thick-smear microscopy and for flaviviruses and alphaviruses by multiplex-nested RT-PCR (7). The samples negative for both malaria and other arboviruses were further tested for OROV with primers targeting the S segment of the OROV genome in a seminested RT-PCR strategy (R.V.M. Bronzoni et al., unpub. data; primers and protocol available from the authors by request). The sample also was isolated in Vero cells, and the RT-PCR described by Moreli et al. (8) was used for confirmation.

We sequenced amplicons by using the same primers used for RT-heminested amplification and by using BigDye Terminators version 3.1 (ABI, Foster City, CA, USA) in ABI377 automated sequencer. Sequences were edited by DSGene 2.0 (Accelrys, San Diego, CA, USA) and deposited in GenBank (accession no. EU561644). One (1.4%) of 69 samples tested for OROV by heminested PCR was positive. This sample (BR/2004/ACRE27) was collected from a male patient from a rural area in April 2004. Precautions were followed to avoid contamination; positive and negative controls were used in all reactions; and the procedure was reproduced several times. The patient had ill-defined, mild flu-like symptoms; low-grade fever; and nasal discharge but reported no headache or other major symptoms. He recovered without complication.

We built a phylogenetic tree on the basis of the 522 nucleotide sequences (27–200 aa) of nucleocapsid protein gene of OROV sample BR/2004/ACRE27 and other GenBank sequences from different OROV genotypes. We used sequences from Aino, Akabane, and Tinaroo viruses as the outgroup. A phylogenetic analysis was performed by the neighbor-joining method by using the Kimura 2-parameter nucleotide substitution model (9).

The tree showed 3 main clades, corresponding to genotypes I, II, and III, and BR/2004/ACRE27 grouped within genotype I strains (Figure). Both genotypes I and II have been described in OROV outbreaks in Acre; genotype I, however, is found mostly in Pará in the eastern part of the Brazilian Amazon region.

A baseline serologic survey in rural Acrelândia during March and April 2004 detected antibodies to OROV in 6 (1.7%) of 357 persons 5–90 years of age who were examined by microplaque hemagglutination inhibition (10). Because none of these persons had been exposed to known OROV outbreaks in Acre or elsewhere, these findings further suggest the sporadic circulation of OROV in the area.

We describe a sporadic infection of OROV infection in the Amazon region of Brazil in a mildly symptomatic patient. The nucleocapsid gene of the isolate has been sequenced, placing it in the genotype I group, the most commonly found in the Amazon Basin. These data suggest that OROV circulation may be sporadic and clinically

Figure. Phylogenetic tree of Oropouche virus strains; **boldface** shows the sample from the patient in this study. Phylogenetic tree was constructed from partial nucleocapsid gene sequence (522 nt, 27–200 aa) by neighbor-joining method implemented in MEGA 3.0 software (9). Kimura 2-parameter nucleotide substitution model was used, and the reliability of the branching patterns was tested by 1,000 bootstrap pseudo replicates. Bootstrap values (%) are shown in main nodes. *Aino*, *Akabane*, and *Tinaroo* viruses were used as the out group. The scale bar represents 5% nucleotide sequence divergence. GenBank accession numbers are provided and are grouped by strain designation. GI, genotype I; GII, genotype II; GIII, genotype III.

silent and, when not associated with outbreaks, most likely neglected by local physicians.

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Meningitis Caused by *Streptococcus suis* Serotype 14, North America

To the Editor: *Streptococcus suis* is an opportunistic pathogen that can cause serious systemic infections in pigs and occupation-related infections in humans who work in close contact with pigs or pork by-products. Most *S. suis* organisms isolated from diseased pigs belong to serotypes 1–8 (*1*). The most prevalent strain worldwide is serotype 2, which causes invasive infections in pigs and humans (*2*). We report a case of human meningitis caused by *S. suis* serotype 14.

The patient was a 59-year-old woman from rural Manitoba, Canada; she worked at a hog plant and handled 300–400 piglets/day. In October 2007, when she sought care, she had a 2-day history of fever, vomiting, headache, neck pain, and reduced consciousness. She was febrile and confused and had meningeal signs. Leukocyte count was 19,900/mm^3. Cerebrospinal fluid (CSF) had 284×10^6/L leukocytes (59% lymphocytes, 41% polymorphonuclear cells), 2.3 mmol/L glucose, and 1.85 g/L total protein. Gram stain of CSF showed gram-positive cocci in pairs; cefotaxime and vancomycin were prescribed empirically. Results of computed tomography of the head, chest radiograph, and transesophageal echocardiogram were within normal limits. Blood culture was negative after 5 days of incubation. The CSF culture grew small α-hemolytic colonies on blood agar and chocolate agar. The organisms were gram-positive cocci in chains, were catalase negative, and were identified as *S. suis* by Vitek II and API 20 Strep System (both from bioMérieux, St.-Laurent, Quebec City, Canada).

Identification of the organism as *S. suis* was confirmed at the National Microbiology Laboratory, Winnipeg, Manitoba, Canada, by conventional

biochemical tests (*3*), the results of which were consistent with that of the type strain (Table) and were also confirmed by 16S rRNA gene sequencing, which showed 100% homology with the *S. suis* type strain ATCC 43765, GenBank accession no. EU 477176.

Antimicrobial-drug susceptibilities were determined by microbroth dilution by using Sensititre STP3F panels (Nova Century Scientific Inc., Burlington, Ontario, Canada) and cation-adjusted Mueller Hinton broth with lysed horse blood (2%–5% vol/vol) by TREK Diagnostic Systems, Inc. (Nova Century Scientific Inc.) using manufacturer's instructions and following Clinical and Laboratory Standards Institute guidelines for *Streptococcus* spp. other than *S. pneumoniae* (*4*). This isolate was sensitive to penicillin, cefepime, cefotaxime, ceftriaxone, linezolid, trimethoprim and sulfamethoxazole, vancomycin, meropenem, and levofloxacin; it was resistant to azithromycin, erythromycin, and tetracycline. The isolate was sent to the International Reference Laboratory at the Université de Montréal, Montréal, Québec, Canada, for *S. suis* serotyping, where it was identified by the coagglutination test as serotype 14 (*5*).

The patient recovered quickly, and her therapy was changed to penicillin G. She was transferred to her local hospital to complete her medication. Within a week of her initial visit, bilateral deafness and loss of balance developed and progressed over the next month and had not ameliorated after 1 year.

Human *S. suis* infections result primarily from direct contact (with wounds on skin or mucosa of the mouth and nasal cavity) with carrier pigs, sick pigs, or raw pork contaminated with *S. suis* (*2*). The infection rate among abattoir workers, pig breeders, meat processing workers, and veterinarians is ≈3 cases/100,000 (1,500× higher than the rate for the general population) (*2*). A striking sequela to *S. suis* meningitis is deafness or vestibular dysfunction (*2*,*6*). A consistently higher percentage

of persons experienced deafness after *S. suis* infection than after infection with other meningitis-causing bacteria, 50% and 65% in Europe and Asia, respectively (*2*).

Most cases of *S. suis* infection in humans have been attributed to serotype 2 strains. Only 4 human cases have been reported in North America: 2 in Canada (1 endocarditis, 1 meningitis) and 2 cases of meningitis in the United States (*6–9*). All 4 cases were attributed to *S. suis* serotype 2. Serotype 14 has been reported as a human

pathogen in the Netherlands, Thailand, the United Kingdom, and Denmark and has been routinely isolated from diseased pigs in Canada (*10*).

Although in pigs the organism is present in the upper respiratory tract, particularly the tonsils, nasal cavities, genital tract, and alimentary tract, the mode of transmission to humans reported so far had been through cuts in the hands. Our patient handled hundreds of piglets every day and most likely acquired the infection through her hands. Her meningitis was com-

Table. Identification of organism isolated from cerebrospinal fluid of 59-year-old woman with meningitis, Manitoba, Canada*

Test	*Streptococcus suis* (*3*)	Patient isolate
α-hemolysis on sheep blood agar	+	+
Motility	–	–
Catalase	–	–
Oxidase	ND	–
Fermented		
L-arabinose	–	–
D-glucose	+	+
Glycerol	–	–
Inulin	+	+
Lactose	+	+
Maltose	+	+
Mannitol	–	–
Melezitose	–	–
Melibiose	Variable	+
Raffinose	Variable	+
Ribose	–	–
Salicin	+	+
Sorbitol	–	–
Sucrose	+	+
Trehalose	+	+
Hydrolyzed		
L-arginine	+	–
Esculin/bile esculin	+/ND	±
Starch	+	+
Glycogen	+	+
Hippurate	–	–
Acetoin	–	–
Optochin disk	Resistant	ND
Enzymes		
α-galactosidase	+	+
β-galactosidase	Variable	+
β-glucuronidase	+	+
Leucine arylamidase	+	+
N-acetylglucosaminidase	+	+
Acid phosphatase	–	–
Alkaline phosphatase	–	–
Pyrrolidonylarylamidase	–	–
API Strep code		4640473 high degree (97%) confidence *S. suis*

*+, positive; –, negative; ND, not done; API Strep code, API 20 Strep, API System (bioMérieux, St.-Laurent, Quebec City, Canada).

plicated by bilateral hearing loss with vestibular dysfunction. Preexisting medical conditions, such as alcoholism, liver cirrhosis, or splenectomy, have been described to predispose patients to severe infection and hearing loss (*2*). Our patient, however, did not have any predisposing conditions.

Meningitis in humans caused by *S. suis* serotype 14 is less common than that caused by serotype 2, but the consequences are similar and can be reduced by early treatment with antimicrobial drugs. Identifying this case of meningitis caused by *S. suis* serotype 14 in Canada raises concerns about the public health aspect of this infection. Guidelines may be required to ensure that staff working in hog plants are aware of the risk for this infection and that they use adequate personal protective equipment.

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Outbreaks Caused by New Variants of *Vibrio cholerae O1* El Tor, India

To the Editor: *Vibrio cholerae* O1, the causative agent of cholera, has 2 biotypes (classical and El Tor), which have traditionally been distinguished by phenotypic tests and by genetic differences in the major toxin-coregulated pilus (TCP) gene, the *tcpA* allele of the TCP cluster (*1*), the *rstR* region (regulatory region for phage lysogeny) of CTX phages (*2*), the type of cholera toxin (CT) produced, and the infection pattern of the disease they cause. However, 3 variants of the El Tor biotype have been described recently: Matlab (a place in Bangladesh) variants in 2002 (*3*), which could not be biotyped because they have a mixture of both classical and El Tor (*4*), Mozambique variant in 2004–2005, which has a typical El Tor genome but a tandem repeat of the classical CTX prophage in the small chromosome (*5*), and the altered El Tor type (a typical El Tor biotype and an El Tor CTX prophage that produces CT of the classical type) predominant in Bangladesh since 2001 (*6*). Hybrid vibrios have also been described in other regions of Asia and Africa (*7*).

CT, encoded by the *ctxA* and *ctxB* genes, is the principal toxin produced by *V. cholerae* O1 and O139. Methods for differentiating the biotype-specific CT-B subunit of *V. cholerae* O1 include sequencing the *ctxB* gene, performing an ELISA wth a monoclonal antibody specific to the classical or El Tor CT, or by using a mismatch amplification mutation assay (MAMA)–PCR to distinguish between 2 kinds of *ctxB* genes. This assay detects sequence polymorphisms based on nt position 203 of the *ctxB* gene (*8*).

In Punjab and Haryana states of northern India, during July–September 2007, 6 clusters of cholera outbreak were identified. A total of 745

case-patients were admitted to local government hospitals; the cholera attack rate was 183/1,000 population. Four deaths were reported (case-fatality rate 0.5%). The number of cases per cluster varied from 15 to 400, and adults were primarily affected (74%); 20% of patients had severe dehydration. *V. cholerae* O1 Ogawa was confirmed from stool cultures by using standard isolation, biochemical, and serotyping methods. Twenty-six isolates were phenotypically and genotypically characterized according to biotype. Phenotypic characterization included the Voges-Proskauer reaction, polymyxin B (50 U) susceptibility, chick cell agglutination, and sheep erythrocyte hemolysis; all isolates were confirmed as El Tor biotype. Genotypic characterization included PCR assays for *ctxA* and *tcpA* (*2*), *rstR* (*3*), and *ctxB* (*8*). All strains were toxigenic because each carried the *ctxA* gene. All strains also carried El Tor–specific *tcp*A (472 bp) and *rst*R genes (500 bp*)*. MAMA-PCR showed the *ctxB* gene of both El Tor and the classical type in 21 (80%) of 26 isolates tested.

Similarly, we also tested 20 available isolates from the 2002 outbreak and 4 and 19 sporadic isolates from 2003 and 2004, respectively; all were phenotypically and genotypically confirmed as El Tor and had only *ctxB* of the El Tor type. Of 53 water samples tested during the 2007 outbreak, 4 grew *V. cholerae*. Three samples were confirmed to be non-O1, non-O139 strains. Only 1 isolate was *V. cholerae* O1, which was positive for tcp, *ctxA*, and *ctxB* of both classical and El Tor types (Table).

During the cholera outbreak of 2002 in Chandigarh (*9*), only 1 death was reported (case-fatality rate, <0.01%); the attack rate was 20/1,000, 58.6% were children, and only 10% had severe dehydration. Before the most recent outbreak, the affected regions of Panjab and Haryana (Ambala, Nurpur, Kurali, Mohali, Panchkula, and Raili) had been free of cholera outbreaks since 1994, though sporadic cases had been reported. The 4 deaths from cholera in 2007, along with adult preponderance, high attack rate, more severe illness, and 6 different clusters, point towards a change in the disease's epidemiology. This change may be related to circulation of the hybrid vibrios in this region. In Bangladesh, all strains of *V. cholerae* O1 examined since 2001 belong to the altered El Tor type (*6*), which produces CT of the classical type. This altered type has replaced the seventh pandemic strain of the El Tor biotype that produced CT of the El Tor type, which indicates that a cryptic change has occurred in the seventh pandemic El Tor biotype strains of *V. cholerae* O1.

Newly emerged variants from Bangladesh (*8*) have the genetic makeup of El Tor with *ctxB* gene of only classical, whereas our strains are unique in having *ctxB* of both the clas- sical and El Tor biotypes. Our strains appear to be different from the Mozambique variant *V. cholerae* O1 (*10*), which has *rstR* of the classical type, in that our strains have *rstR* of only the El Tor type. Of 5 Matlab variants analyzed with MAMA-PCR, 3 had classical *ctxB* and 2 had El Tor type. Our study highlights the different genetic recombinations possible in *V. cholerae* and the epidemiologic role of these recombinations.

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Table. Phenotypic and genotypic traits of *Vibrio cholerae* O1 clinical strains isolated from northern India, 2002–2007*

Year of isolation or type of strain	Phenotypic tests				PCR amplicons		
	VP	Polymyxin B (50 U) susceptibility	CCA	Sheep erythrocyte hemolysis	*tcpA*	*ctxB* (MAMA-PCR)	*rstR*
2002 (n = 20)	+	Resistant	+	+	E	E	E
2003 (n = 4)	+	Resistant	+	+	E	E	E
2004 (n = 19)	+	Resistant	+	+	E	E	E
2007 (n = 26)	+	Resistant	+	+	E	E + C (n = 21)†	E
Environmental (n = 1)	+	Resistant	+	+	E	E + C	E
Classical 569B	–	Sensitive	–	–	C	C	C
El Tor N16961	+	Resistant	+	+	E	E	E
Hybrid NICED, India	+	Resistant	+	+	E	C	E + C

*VP, Voges-Proskauer; CCA, chick cell agglutination; MAMA, mismatch amplification mutation assay; +, positive; –, negative; E, El Tor type; C, classical type; NICED, National Institute of Cholera and Enteric Diseases, Kolkata, India.
†Remainder had *ctxB* of El Tor type.

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Human Case of *Atopobium rimae* Bacteremia

To the Editor: The genus *Atopobium* (*1*) accommodates species formerly designated *Lactobacillus minutus*, *L. rimae,* and *Streptococcus parvulus* (*2*). Use of 16S rDNA sequence analysis showed these species to be closely related and to form a distinct line of descent within the lactic acid bacteria (*3*). *Atopobium* spp. usually have been recognized as part of the human gingival oral flora; some species, including *A. rimae* and *A. parvulum*, have been identified as agents of chronic periodontitis (*4,5*). *A. rimae*, formerly known as *L. rimae* (*1*), forms short, gram-positive, strictly anaerobic, elliptical bacteria with low G+C content (*4*). *A. rimae* produces large amounts of lactic acid and has been recovered previously from normal human gingival flora (*4,5*). Apart from periodontitis, it has not been implicated in other types of infection. We report an unusual case of *A. rimae* bacteremia.

In May 2007, a 77-year-old woman with a history of right thoracotomy for pneumothorax 2 years earlier was hospitalized for inhalation pneumonia caused by paralysis of the right vocal cord. During hospitalization, septic shock and a fever of 38°C developed in the patient, complicated by acute respiratory failure and stroke. She was transferred to an intensive care unit with a PaO_2/FiO_2 >300 mm Hg, and a tracheotomy was performed. Three anaerobic blood specimens, drawn at entrance into the intensive care unit, yielded gram-positive cocci after 24-h incubation of the first bottle and gram-positive bacilli after 48-h incubation of the 2 other bottles. The gram-positive cocci were identified as *Streptococcus gordonii* using API STREP (bioMérieux, Marcy l'Etoile, France). The gram-positive bacilli were catalase negative and oxidase positive

but remained unidentified with use of API ANA strip (bioMérieux). Minimum inhibitory concentrations of antibiotics were determined for the gram-positive bacilli using E-test assay (AB BIODISK, Solna, Sweden) on Columbia agar supplemented with 5% sheep blood. Minimum inhibitory concentrations were 0.064 μg/mL for penicillin G, 0.023 μg/mL for ampicillin, 0.012 μg/mL for amoxicillin–clavulanic acid, 0.032 μg/mL for imipenem, <0.016 μg/mL for azithromycin, <0.016 μg/mL for erythromycin, 0.06 μg/mL for ciprofloxacin, and 1.25 μg/mL for vancomycin. DNA was extracted from 1 colony by using a QIAamp tissue kit (QIAGEN, Hilden, Germany) as described by the manufacturer. The 1,454-bp 16S rDNA sequence obtained using the fD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and rP2 5′-ACGGCTACCTTGTTACGACTT-3′ primer pair (*6,7*) showed 99% sequence similarity with the 16S rDNA sequence of *A. rimae* (GenBank accession no. AF292371) by use of BLAST version 2.2.9 software (National Center for Biotechnology Information). A phylogenetic neighbor-joining tree based on the *Atopobium* spp. 16S rDNA sequences made with the MEGA software confirmed that the isolate belonged to *A. rimae* (Figure). Initial treatment by intravenous tazocilline-amikacin was changed to intravenous amoxicillin–clavulanic acid (2 g/200 mg). The fever resolved, and the patient's condition improved. The treatment was stopped after 7 days, and the patient remained apyretic.

In this case, phenotypic identification of gram-positive bacillus isolated from 2 blood cultures failed because the definite bacterial species *A. rimae* was not included in the API database used for the phenotypic identification. Final identification was achieved within 2 days by comparison of the almost complete 16S rDNA sequence with homologous sequences deposited in Genbank. This comparison yielded a 99% sequence similarity, regarded
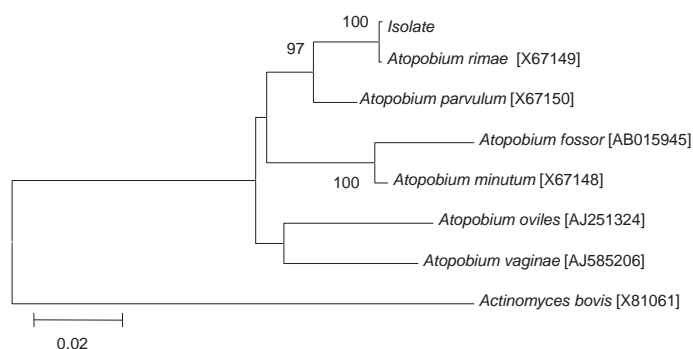
Figure. 16S rDNA maximum-likelihood phylogenetic tree showing the relationships of a blood isolate with *Atopobium* species. GenBank accession numbers are indicated in brackets. 16S rDNA sequence of *Actinomyces bovis* was used as an outgroup. Bootstrap values >90% as indicated at nodes. Scale bar indicates 0.02 substitutions per nucleotide position.

as criteria for accurate identification of bacterial organisms at the species level (*8*). In this patient, 2 *A. rimae* isolates were recovered from 2 different blood-culture bottles drawn 48 h apart, suggesting that *A. rimae* was not just a bypassing organism but indeed responsible for septicemia. In these specimens, *S. gordonii* was also isolated. Both species have been described as belonging to the oral flora, suggesting that these flora probably were the source for mixed septicemia in the patient. *A. rimae* was isolated as the patient was presenting with clinical features of septic shock, suggesting that *A. rimae* may have contributed to the shock. Antimicrobial drug treatment based on in vitro *A. rimae* susceptibility profile, along with reanimation measures, allowed for the patient's recovery.

This case report illustrates the usefulness of 16S rDNA sequencing for accurate identification of anaerobic organisms and suggests that *A. rimae* should be added to the list of organisms responsible for bacteremia in patients.

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Systemic Infection with Enteric Adenovirus in Immunocompetent Child with *Haemophilus influenzae* Disease

To the Editor: Recent articles have reported enteric human adenoviruses (HAdVs) types 40 and 41, previously thought to be restricted to the gastrointestinal tract (*1*), in multiple organ systems of a deceased immunodeficient child (*2*) and in respiratory specimens of children with acute respiratory illnesses (*3*). Here we present a case in which enteric HAdV-40 was found in the cerebrospinal fluid (CSF) and blood of an apparently immunocompetent child with *Haemophilus influenzae* invasive disease.

The patient, a 10-month-old previously healthy Thai boy, met the criteria for a clinical case of encephalitis (*4*) and, after informed consent was obtained, was enrolled in the study of causes of encephalitis in Thailand (collaboration between the US Cen-

ters for Disease Control and Prevention [CDC] and the Ministry of Health of Thailand). Clinical and laboratory information was collected from the medical record. Biologic specimens were sent to CDC and to the Thailand National Institute of Health, Nonthaburi, Thailand, for extensive testing for a broad range of pathogens potentially associated with encephalitis (*4*). Data on the clinical course of the patient are presented in the Table.

HAdV DNA was first detected in the CSF specimen collected on December 12, 2003, by an in-house pan-AdV PCR screening assay conducted as part of the study protocol. Amplicon sequences obtained closely matched that of HAdV-40. This unexpected result was confirmed by independent PCR assays on separate aliquots of the same specimen, a broadly reactive real-time TaqMan PCR targeting the hexon gene, and a HAdV 40/41 type-specific real-time Förster resonance energy transfer (FRET) PCR assay targeting the fiber gene (*5*). Sequences of the hexon gene hypervariable regions 1–6 that provide type specificity

(*6*) showed a single nonsynonymous base substitution (C→T; Thr→Ile) at nucleotide position 107 of the HAdV-40 prototype strain Dugan (GenBank accession no. DQ115441).

HAdV-40 DNA with identical sequences was also detected in the acute-phase serum specimen also collected on December 12, 2003, but not in the convalescent-phase specimen collected on January 7, 2004. An oropharyngeal swab specimen obtained on December 12 was PCR-negative for HAdV DNA. Although no increase in levels of HAdV antibodies was detected by indirect enzyme immunoassay against pan-AdV antigen, microneutralization assay demonstrated a rise in levels of type-specific neutralizing antibodies to HAdV-40 between the acute-phase (<1:10) and convalescent-phase (1:40) serum specimens.

The results of other testing conducted on the same specimens as part of the study protocol were the following. CSF obtained on December 12, 2003, was negative by broad-specificity PCRs for bacterial 16S RNA and viral agents (alphaviruses, flaviviruses, bunyaviruses, human herpesviruses) as well as by PCR for enteroviruses, herpes simplex virus, Nipah virus, *Mycoplasma pneumoniae*, and *Neisseria meningitidis.* CSF was also negative for *Cryptococcus* spp. by India ink technique. Serum was negative for acute infection with flaviviruses (dengue and Japanese encephalitis viruses); alphaviruses (chikungunya virus); influenza viruses; human parainfluenza viruses 1–3; measles, mumps, and rubella viruses; enteroviruses; *Bartonella henselae;* rickettsiae (*R. typhi, Orientia tsutsugamushi,* and *R. conorii*); and *M. pneumoniae.* Results of PCR on saliva specimens and serologic testing for rabies were negative; an oropharyngeal swab specimen was negative by PCR for *M. pneumoniae*; and results of a smear for malaria parasites were negative. The patient was HIV negative.

Detection of HAdV-40 in CSF in this case was confirmed by multiple PCRs with amplicon sequencing. Detection of virus in the acute-phase serum specimen confirms systemic infection and demonstrates that HAdV-40 DNA found in CSF did not arise from contamination of the CSF at the time of collection. Laboratory contamination is also unlikely because the nucleotide sequence of the identified strain (GenBank accession no. FJ228470) was not identical to the prototype reference strain used for positive control in the PCR. Seroconversion to HAdV-40 provides further evidence that this child experienced an acute systemic infection with this virus.

The contribution of HAdV-40 to the clinical illness in this patient remains unclear. He had a confirmed *H. influenzae* invasive infection, which likely explains the initial underlying illness. However, the detection of HAdV-40 coincided in time with the development of neurologic signs (new-onset seizures, ataxia) and widespread rash. By then, the patient had been receiving antimicrobial drug therapy for several days, his

Table. Clinical course of illness in 10-month-old boy with systemic infection with enteric adenovirus and *Haemophilus influenzae* disease, Thailand, 2003–2004*

Date, 2003	Events
Dec 7	Patient hospitalized with 6-day history of fever >38°C and somnolence; blood culture positive for *Hemophilus influenzae*; isolate not typed (unavailable for further characterization)
Dec 9	CSF results: pleocytosis (2,710 leukocytes/mm^3, 94% neutrophils); protein 178 mg/dL; glucose 11 mg/dL; CSF culture positive for *H. influenzae;* CSF Gram stain positive for gram-negative coccobacilli; antimicrobial drug treatment (ceftriaxone) started
Dec 11	New onset seizures, ataxia, and maculopapular rash on entire trunk and all extremities; no diarrhea or respiratory symptoms; brain ultrasound scan results within normal limits; anticonvulsant therapy (phenobarbital) started
Dec 12	CSF results: pleocytosis (100 leukocytes/mm^3, 60% neutrophils, 40% monocytes); protein 131.6 mg/dL; glucose 31 mg/dL; CSF bacterial culture, results negative; CSF Gram stain results negative; patient enrolled in the encephalitis study; initial specimens for the study collected
Dec 22	Brain ultrasound scan results within normal limits; antimicrobial drug treatment (ceftriaxone) discontinued
Dec 23	CSF bacterial culture results negative; CSF Gram stain results negative; anticonvulsant therapy (phenobarbital) discontinued
Dec 27	Patient discharged in improved condition; discharge diagnosis: *H. influenzae* meningitis and septicemia
Jan 7†	Follow-up visit: full recovery without sequelae; convalescent-phase serum specimen obtained

*CSF, cerebrospinal fluid.
†2004.

CSF was negative for 16S bacterial RNA by PCR and culture-negative for *H. influenzae*, and the CSF pleocytosis had decreased substantially. These circumstances make it less likely that these signs were associated with the underlying *H. influenzae* disease and raise the possibility that superimposed HAdV-40 infection played a role. Because the patient had no diarrhea or respiratory symptoms, no evidence of immunodeficiency, no stool specimen available for testing, and no evidence of HAdV in throat swab specimen, the pathogenesis of HAdV-40 infection in this case is unknown. The origin of the maculopapular rash concurrent with neurologic symptoms in this patient is also unclear. Rash is not typical for *H. influenzae* infection and, although reported for some HAdV infections (*7*), has not been previously described for HAdV-40/41.

In conclusion, this case demonstrates the possibility of nongastroenteric, systemic infection involving CNS with enteric HAdV in immunocompetent hosts. Broad-specificity AdV PCR assay followed by amplicon sequencing enabled detection of this pathogen in an unexpected context and can be useful in defining the nongastroenteric disease effects associated with the enteric HAdVs.

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Mesotherapy-associated Outbreak Caused by *Mycobacterium immunogenum*

To the Editor: Mesotherapy, a procedure for medical and cosmetic treatment, involves use of microinjections of different biologically active substances into the dermis or subcutaneous adipose tissue. This controversial practice is used for spot contouring and anti-aging therapy. Concerns have been raised about mesotherapy complications, such as aseptic subcutaneous necrosis and cutaneous nontuberculous mycobacterial infections. Several rapidly growing mycobacterial species, primarily *Mycobacterium fortuitum, M. peregrinum, M. chelonae*, *M. abscessus*, *M. simiae*, and the newly described *M. massiliense*, *M. bolletii*, and *M. cosmeticum* (*1–5*), have been reported to cause infections and outbreaks originating from use of contaminated injectable solutions or skin antiseptics during mesotherapy and other invasive cosmetic procedures. We describe a mesotherapy-

associated outbreak involving an organism compatible with the novel *M. immunogenum* that Wilson et al. first described (*6*).

During September 2006–May 2007, 169 persons underwent mesotherapy at a private aesthetic clinic in the city of Buenos Aires, Argentina. For 28 (17%) skin lesions developed at the injection sites. Patients had been injected during the first 2 months of 2007 with phosphatidylcholine and ampelopsine after receiving a topical antiseptic containing lapyrium chloride from a commercial supplier. The clinic and all the products used in the procedure had been licensed by the national regulatory authority. As soon as the outbreak became evident, the antiseptic solution in use was discarded; no additional cases occurred. By the time the investigation was conducted, these solutions were no longer available for culture.

Nineteen patients were referred to our hospitals. Physical examination found 2–20 nodules 0.5–4 cm in diameter per patient. Lesions were localized on legs (18 patients), buttocks (16), abdomen (1), and forearms (1) and had appeared 7–37 days (median 31 days) after the injection. Occasionally, some nodules produced a serous or purulent discharge, but secretions were not submitted for bacteriologic analysis.

Nodule biopsy was performed for 10 patients, and specimens were sent for histologic and bacteriologic investigations. Histologic examination demonstrated abscesses, areas of fat necrosis, and peripheral fibrous changes. Specimens from 3 patients produced acid-fast bacilli growth in blood agar, mycobacteria growth indicator tube, and Lowenstein-Jensen culture media. When subcultured at 3 different temperatures for identification, all 3 cultures grew preferentially at room temperature rather than at 37°C and 42°C. The isolated mycobacteria were nonpigmented, rapidly growing, and fastidious. The results of

biochemical tests produced a hybrid pattern between *M. chelonae* and *M. abscessus.* Specifically, the isolates were unable to use citrate as the sole carbon source and to grow in the presence of 5% NaCl.

PCR-restriction analysis (PRA) of a 439-bp segment of the *hsp*65 gene digested with *Bst*EII and *Hae*III was performed at the national reference laboratory for tuberculosis of the Instituto Malbran. Species was assigned according to the PRASITE website (http://app.chuv.ch/prasite). The profile of the 3 isolates fit the pattern of *M. immunogenum* type 2 as first described by Sampaio et al. (*7*); the isolates had 325- and 130-bp bands after *Bst*EII digestion and 200-, 70-, 58-, and 55-bp bands after *Hae*III digestion. This is our first detection of this particular PRA profile in Argentina since we started systematic *hsp*65 PRA typing to identify mycobacteria in clinical isolates in 2005. Enterobacterial repetitive intergenic consensus PCR patterns of the 3 isolates were indistinguishable from each other

and differed from epidemiologically unrelated clinical isolates of the *M. abscessus–M. chelonae* group, confirming the clonality of the 3 strains (Figure) (*7*).

Susceptibility to antimicrobial agents was determined by using standard Clinical and Laboratory Standards Institute broth microdilution method (*8*). Clarithromycin, ciprofloxacin, cefoxitin, doxycycline, amikacin, tobramycin, and imipenem MIC values for the 3 isolates were <0.125, 1, 32, >32, 32, 16, and 64 μg/mL, respectively. The disk elution method produced similar patterns of activity for the first 4 antimicrobial agents and inconsistent results for the remaining 3. Patients received a combination of clarithromycin and either ciprofloxacin or levofloxacin for 6–8 months. All 19 cases resolved favorably, although multiple pigmented retractile scars persisted after treatment.

M. immunogenum was identified as the etiologic agent of a variety of hospital-acquired infections, including an outbreak of keratitis, and as
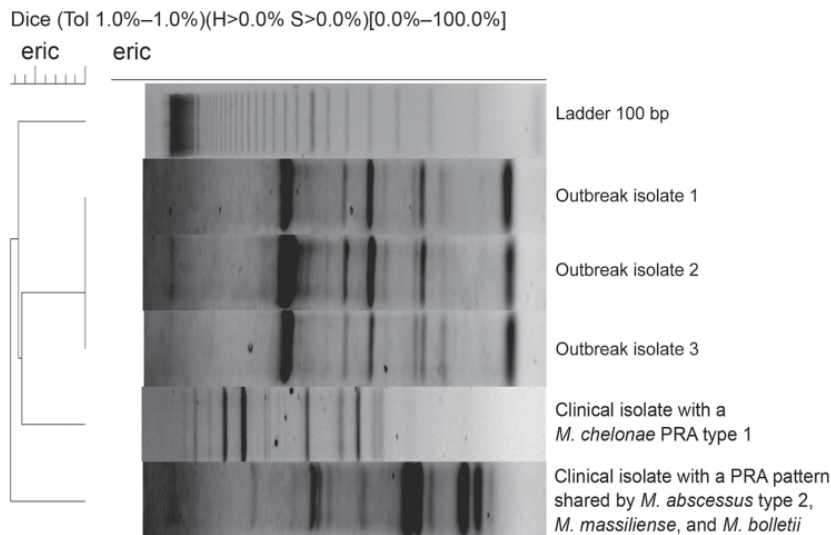
Dice (Tol 1.0%–1.0%)(H>0.0% S>0.0%)[0.0%–100.0%]

Figure. DNA enterobacterial repetitive intergenic consensus PCR (eric) analysis of rapidly growing mycobacteria isolated from 3 patients in a mesotherapy-associated outbreak, January–February, 2007, Buenos Aires city, Argentina, compared with profiles of epidemiologically unrelated clinical isolates of the *Mycobacterium abscessus–M. chelonae* group. The dendrogram was constructed with the aid of BioNumerics software version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium), using Dice unweighted pair group method coefficients with 1% tolerance. PRA, PCR-restriction analysis of the hsp65 gene.

the potential cause of hypersensitivity pneumonitis in industrial metal-grinding machinists (*6,7,9*). This microorganism appears to differ from other members of the *M. chelonae–abscessus* group by subtle mutations in *rpo*B, *hsp65*, the hypervariable region of 16S rRNA, and other housekeeping genes (*5–7,9,10*). The value of minor polymorphisms might be arguable for defining new species and for clinically managing patients. However, because of its rarity among clinical isolates in our country, the PRA type ascribed to *M. immunogenum* proved to be a useful epidemiologic marker to investigate this outbreak.

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Contagious: Cultures, Carriers, and the Outbreak Narrative

Priscilla Wald

The outbreak narrative, which tells the evolving story of disease emergence, is the central theme of this book by Priscilla Wald, an English professor at Duke University. Wald discusses and challenges outbreak narratives that use a formulaic plot line: identification of an emerging infection, discussion of global networks through which diseases travel, and a chronicle of the epidemiologic work that results in disease containment. The expectation created by that formula, Wald contends, has at times hampered our ability to address such emerging diseases as HIV infection because we distort or exaggerate the story to fit the formula. Possibly more important, she claims, is the development of the outbreak narrative in the late 19th century, which coincided with emergence of urban sociology and the concept of social contagion. During this time, circulation of ideas and attitudes turned individuals into social groups and cultures, and terms such as contagion and infection entered lay language.

Wald describes Mary Mallon, "Typhoid Mary," as the prototypical healthy carrier whose outbreak narrative led to a new way of thinking about social relationships and social responsibility. Her status as an immigrant contributed to society's blaming and stigmatizing immigrants because of their association with communicable disease (especially venereal disease), a stigma reanimated in the 1980s with the emergence of HIV and its most infamous carrier, Patient Zero.

Wald's analysis of disease emergence also notes its simultaneous evolution with social, religious, and political changes. The viral metaphor was applied to biologic warfare in the 1950s and expanded to describe political contagion. According to J. Edgar Hoover, "the bloody virus of communism" was spread by agents (carriers) trying to "infiltrate and colonize this country."

The microbe is the worthy foe in epidemiologists' stories, which can be seen as analogous to good detective stories that have happy endings and draw attention to urgent problems. However, Wald warns, what makes the story appealing can distort articulation of the problem. The danger, she believes, lies in the storytelling, not in the epidemiology or laboratory science. Wald also notes that epidemiology's shift away from infectious diseases beginning in the 1960s removed the heroic edge from the field. She writes, "Sociology did not make for risk, exciting disease detectives." Wald is wary of the limitations of the outbreak narrative and advocates a model for global health based on social justice.

The book is academic in presentation, and Wald's conclusions are not always compelling. For example, her discussion of epidemiologic horror in books and movies such as Invasion of the Body Snatchers will intrigue some readers and put off others who might find this analysis extreme and detracting from the serious tone of the rest of the book. Still, the thesis involving the outbreak narrative is interesting, and readers intrigued by the influence of epidemiology on emerging infections as well as its broader implications to society and the world will find this book worthwhile.

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Lois Mailou Jones (1905–1998). Ubi Girl from Tai Region (1972). Acrylic on canvas (111.1 cm × 152.4 cm). Museum of Fine Arts, Boston, Massachusetts USA. The Hayden Collection—Charles Henry Hayden Fund, 1974.410

… Myself That I Remake

—W.B. Yeats

Polyxeni Potter

"I'm a lover of nature. I have to paint from within …. As I used to tell my students, anything I do must be of a caliber that will live after me. That is really my credo, even now," said Lois Jones when she was in her 80s. During an artistic career that spanned more than 6 decades and continued until the end of her life, she sought excellence and a place in art history. When, in her early efforts, she realized that textile design entailed anonymity, she abandoned it. "As I wanted my name to go down in history, I realized that I would have to be a painter. And so it was that I turned immediately to painting."

A Boston native, Jones received her early education locally, first at the High School of Practical Arts and in museum vocational drawing classes, later at the Boston Museum School of Fine Arts and the Designers Art School, where she studied textile design and created patterns for such textile manufacturers as F.A. Foster in Boston and Schumacher in New York City.

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"Practically every summer of my childhood my mother took me and my brother to Martha's Vineyard …. It was there that I first began to paint." During these trips to the island Jones met two influential figures, sculptor Meta Warrick Fuller and composer Harry T. Burleigh. "Lois, you know, if you want to be successful in your career you're going to have to go abroad," they advised. Fuller had studied in Paris with Rodin, and Burleigh would regularly go to Switzerland to write his music. "Look what happened to Henry O. Tanner. He couldn't make it in this country; he had to go abroad. The same thing happened to Hale Woodruff."

Though Jones vowed to go abroad, Paris had to wait. After graduation from the Boston Museum School, she migrated south to North Carolina to teach at the Palmer Memorial Institute in Sedalia. Soon she was invited to Howard University in Washington, DC, where she founded the art department and taught design and watercolor painting for more than 40 years, making her mark and influencing hundreds of painters, among them Elizabeth Catlett, Alma Woodsey Thomas, and Malkia Roberts.

Jones got her big break in 1937, with a fellowship to study at the Académie Julien during a sabbatical from How-

ard. "I began to think of my dream coming true, of going to Paris, where I would be appreciated as an artist … and see the paintings of Lois Jones hanging beside those of artists from all over France and all over the world …." In Paris, she gained confidence, met distinguished colleagues, and exhibited widely. "Impressionism was the way I worked … mostly with the palette knife."

"When I returned from France … I missed all of that elegance which I had known in Paris …." Under the new circumstances, "I owed very much to my white friend Céline who would take my paintings to the juries. They never knew that the artist was black. That was very much in my favor."Jones returned to France often and went on other travels, notably to Africa and Haiti. She became comfortable in many styles and with portraits as well as landscapes. Ubi Girl from Tai Region, on this month's cover, shows the influence of what was known as the Washington Color School, a group that favored precise motifs and bold flat fields of color. At a symposium in 1992, Jones described herself as the "only surviving painter of the Harlem Renaissance," the movement of the 1920s and 1930s that celebrated African cultural identity and heritage.

"I bathed in the Euphrates when dawns were young/I built my hut near the Congo and it lulled me to sleep," wrote Jones' contemporary Langston Hughes, expressing his generation's preoccupation with origins and identity. Jones delved into her own roots early in her career. "I can remember the students at Howard University saying that Professor [James A.] Porter, Professor [James Lesesne] Wells, and I didn't appreciate African art. The students thought that they were the ones who were bringing African art into recognition. I told them that in 1937, before they were born, I had painted Les Fetiches …."

"I found that the African masks gave me my best opportunity for studying the mask as a form, and my interest in the mask began very early in my career…. In Africa, I would go to the museums and make sketches and studies from the fetiches and the masks and use them in my creative paintings." Jones was not alone in her fascination. In the 1900s, African sculpture became a strong force in the development of modern art, even though its original meaning and function were not known. In France, an underlying spiritual content was recognized. Stylized features were adapted and combined with postimpressionist elements from Cézanne and Gauguin to define the flat fragments of Henri Matisse, Pablo Picasso, and other pioneers of modernism.

Picasso described his bewildering first exposure to African masks in 1907. "When I went to the Trocadéro … a smell of mould and neglect caught me by the throat .... But I forced myself to stay, to examine these masks … that

people had created with a sacred, magical purpose, to serve as intermediaries between them and the unknown …. And then I understood what painting really meant. It's not an aesthetic process; it's a form of magic that interposes itself between us and the hostile universe, a means of seizing power by imposing a form on our terrors as well as on our desires. The day I understood that, I realized that I had found my path."

Masks have featured in many disciplines. William Butler Yeats (1865–1939) viewed them in the context of spiritual renewal. Like Picasso, he saw beyond aesthetics. He believed that artists are charged with perfecting themselves and the world and admonished "poet and sculptor" to perform this miracle. He was relentless in his own efforts. "The friends that have it I do wrong/When ever I remake a song," he wrote, "Should know what issue is at stake:/It is myself that I remake."

In many style transformations over her lengthy career, Jones did her part, remaking herself, revising, seeking new levels of perfection. The bold geometric designs of Ubi Girl from Tai Region, far removed from her early impressionist style, capture the underlying spirituality in the faces and masks she studied during her travels.

The Pythian expressions of the masks against the dark profile in the center of the painting challenge the viewer. What horror do they encase? What spirit do they conceal? Perhaps they shield against social injustice as experienced by Jones and so many others. Outside the artist's realm and far from their origins, masks still serve as intermediaries between us and the unknown. In public health, they shield against nature's horrors, respiratory viruses haunting our households. If worn consistently as designed, masks can protect against the flu and other infections. A new face we create for ourselves, they are still a "form of magic" pressed "between us and the hostile universe."

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Meeting the Challenge of Influenza Pandemic Preparedness in Developing Countries

Coccidioidal Pneumonia, Phoenix, Arizona, 2000–2004

Sources of Hepatitis E Virus Genotype 3 in the Netherlands

Avian Influenza Viruses A (H5N1) from Wild Birds, Hong Kong, 2004–2008

Influenza-like Illness in Children, Nicaragua, 2005–2007

Locus of Enterocyte Effacement–negative Shiga Toxin–producing *Escherichia coli*

Capacity of Thailand to Contain an Emerging Influenza Pandemic

Integron-Mediated Multidrug Resistance in Nontyphoidal *Salmonella enterica*

Clinical Risk Factors for Severe *Clostridium difficile*–associated Disease

Epidemiology of Bluetongue Virus Serotype 8, Germany

Seroprevalence of Hepatitis E Virus Antibody in Patients with Chronic Liver Disease

Detection of Novel SARS-like and Other Coronaviruses in Bats from Kenya

Introduction of Distinct Genotype of Avian Influenza Virus A (H5N1) into Nigeria

Border Disease Virus among Chamois, Spain

Merkel Cell Polyomavirus in Respiratory Tract Secretions

**Complete list of articles in the March issue at
http://www.cdc.gov/eid/upcoming.htm**

Upcoming Infectious Disease Activities

February 12–13, 2009
The International Symposium
on the Asian Tiger Mosquito
Rutgers University
New Brunswick, NJ, USA
http://www.rci.rutgers.edu/~vbcenter/
atmsymposium.php

February 13–16, 2009
International Meeting on Emerging
Diseases and Surveillance (IMED 2009)
Hotel Hilton
Vienna, Austria
http://imed.isid.org

February 22–25, 2009
7th American Society for
Microbiology Biodefense
and Emerging Diseases Research
Meeting
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.asmbiodefense.org

April 2–3, 2009
Exploring the Dynamic Relationship
Between Health and the Environment
The Center for Biodiversity and
Conservation Milstein Science
Symposium
American Museum of Natural History
New York City, NY, USA
http://cbc.amnh.org/health
cbcsymposium@amnh.org

April 27–29, 2009
Twelfth Annual Conference
on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org/conferences/
vaccine09

June 28–July 1, 2009
18th ISSTDR: International Society
for STD Research
QEII Conference Centre
London, UK
http://www.isstdrlondon2009.com

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Article Title

Face Mask Use and Control of Respiratory Virus Transmission in Households

CME Questions

1. Which of the following is least likely to be a nonpharmaceutical strategy examined and reported for the prevention of influenza-like infection (ILI) during an influenza pandemic?
A. School closure
B. Use of face masks
C. Handwashing
D. Quarantine at home

2. Which of the following best describes the type of study used to examine the efficacy of face masks in respiratory infection control at home?
A. Retrospective case-control study
B. Prospective cluster-randomized study
C. Prospective case-control study
D. Observational case series

3. Which of the following is the most common single viral respiratory pathogen to be isolated from 141 children with respiratory viral illness in the study reported?
A. Influenza B
B. Influenza A
C. Adenovirus
D. Respiratory syncytial virus

4. Which of the following best describes the adherence rate for P2 face masks on day 5, after beginning the use of face masks by household adult contacts for household infection control?
A. 25%
B. 31%
C. 36%
D. 46%

5. Which of the following best describes the hazard ratio for risk for transmission of ILI if adherence to face mask use was 100%?
A. 1.00
B. 0.85
C. 0.47
D. 0.26

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

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Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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