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On the Cover

Henry Ossawa Tanner (1859–1937)
The Banjo Lesson (1893)
Oil on canvas (124.46 cm x 90.17 cm)
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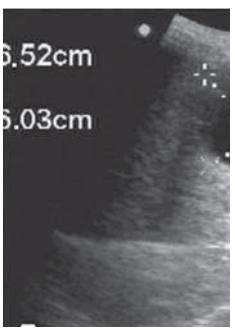
Research



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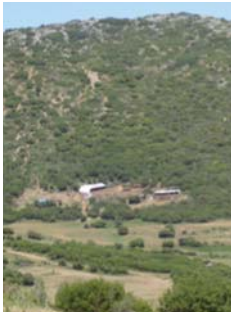

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Dissemination of Clonally Related *Escherichia coli* Strains Expressing Extended-Spectrum β-Lactamase CTX-M-15

Teresa M. Coque,*†‡ Ângela Novais,*†‡ Alessandra Carattoli,§ Laurent Poirel,¶ Johann Pitout,# Luísa Peixe,†† Fernando Baquero,*†‡ Rafael Cantón,*†‡ and Patrice Nordmann¶**

We analyzed 43 CTX-M-15–producing *Escherichia coli* isolates and 6 plasmids encoding the $bla_{CTX\text{-}M\text{-}15}$ gene from Canada, India, Kuwait, France, Switzerland, Portugal, and Spain. Most isolates belonged to phylogroups B2 (50%) and D (25%). An EC-B2 strain of clonal complex sequence type (ST) 131 was detected in all countries; other B2 isolates corresponded to ST28, ST405, ST354, and ST695 from specific areas. EC-D strains were clonally unrelated but isolates from 3 countries belonged to ST405. All CTX-M-15 plasmids corresponded to IncFII group with overrepresentation of 3 *Hpa*I-digested plasmid DNA profiles (A, B and C; 85–120kb, similarity ≥70%). Plasmid A was detected in EC-B2 strains (ST131, ST354, or ST405), plasmid C was detected in B2 and D strains, and plasmid B was confined to worldwide-disseminated ST131. Most plasmids contained $bla_{OXA\text{-}1}$, *aac(6')-Ib-cr*, and $bla_{TEM\text{-}1}$. Worldwide dissemination of CTX-M-15 seems to be determined by clonal complexes ST131 and ST405 and multidrug-resistant IncFII plasmids.

Plasmid-mediated CTX-M type expanded-spectrum β-lactamases (ESBLs), which have been extensively reported for the past 10 years, are detected mostly in community-acquired pathogens and are associated mainly with *Escherichia coli*. These β-lactamases compromise the efficacy of all β-lactams, except carbapenems and cephamycins, and are associated with many non–β-lactam resistance markers because of their locations on plasmids. Therefore, they may constitute a real threat for treating community-acquired *E. coli*–mediated urinary tract infections (*1*,*2*).

Different variants of CTX-M ESBLs are grouped in 5 clusters, although their distribution varies greatly depending on the geographic area (www.lahey.org/studies/webt.htm). CTX-M-15, which was first detected in isolates from India in 2001 (*3*), is now recognized as the most widely distributed CTX-M enzyme. It is derived from CTX-M-3 by 1 amino acid substitution at position 240 (Asp-240 → Gly), which apparently confers an increased catalytic activity to ceftazidime (*4*). Clonal outbreaks of CTX-M-15–producing *Enterobacteriaceae* have been reported in France, Italy, Spain, Portugal, Austria, Norway, the United Kingdom, Tunisia, South Korea, and Canada, and *E. coli* is the most frequently involved species. Within *E. coli*, CTX-M-15–producing strains of the B2 phylogenetic group are commonly found and frequently harbor multidrug resistance and virulence determinants (*5*–*18*).

Plasmids encoding $bla_{CTX\text{-}M\text{-}15}$ have been isolated from clinical isolates in France, Spain, Portugal, the United Kingdom, Canada, India, Pakistan, South Korea, Taiwan, the United Arabic Emirates, and Honduras (*5*–*8*, *10*,*11*,*15*,*19*,*20*). Plasmid characterization, which has only been accomplished for those plasmids from Canada, France, Spain, and the United Kingdom, classified most of them as members of incompatibility group FII (*5*,*7*,*8*,*17*,*19*).

Lack of detailed studies on isolates expressing particular CTX-Ms from different geographic areas has precluded identification of factors involved in recent and worldwide

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spread of specific CTX-M variants. In this article, through analysis of the population biology of CTX-M-15–producing isolates from 7 countries and characterization of their genetic elements, we provide a comprehensive picture of elements involved in international spread of a particularly widespread mechanism of antimicrobial drug resistance.

Materials and Methods

Bacterial Strains, Production of ESBL, and Susceptibility Testing

We studied 43 CTX-M-15–producing *E. coli* clinical isolates from France (n = 17), Kuwait (n = 9), Switzerland (n = 7), Canada (n = 4), Portugal (n = 3) and Spain (n = 3), and 6 CTX-M-15 plasmids from India (*3*), all obtained from 2000 through 2006. These strains and plasmids were considered representative of these areas because they either caused outbreaks or were the first isolates recovered in those countries (*3*,*11*,*16*,*19*,*21–23*). Samples were isolated from urine (n = 33/43, 77%), wounds (n = 4/43, 9.%), respiratory tract infections (n = 3/43, 7%) and other sites (1 from feces, 1 from an intravenous catheter, and 1 from blood) in hospitalized patients. ESBL production was confirmed by a standard double-disk synergy test, and *bla* genes were characterized by PCR and additional sequencing as described (*19*). Susceptibility patterns to 13 non–β-lactam antimicrobial drugs were determined by the standard disk diffusion method following published standards (*24*). Strains with intermediate susceptibility were considered resistant.

Clonal Relationships

Clonal relationships were established by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested genomic DNA (New England Biolabs, Ipswich, MA, USA) as described (*25*). Assignment of *E. coli* phylogenetic groups was conducted by using a multiplex PCR assay described by Clermont et al. (*26*). All *E. coli* isolates belonging to phylogroups B2 and D were characterized by multilocus sequence typing (MLST) using the standard 7 housekeeping loci (www.mlst.net). All *fumC* sequences from *E. coli* isolates belonging to phylogroup D were analyzed for a C288T single nucleotide polymorphism. This polymorphism is specific for a globally disseminated *E. coli* strain arbitrarily designated as *E. coli* clonal group A (CgA) that is associated with community-acquired urinary tract infections (*27*,*28*).

Transferability and Location of $bla_{CTX\text{-}M\text{-}15}$

Transferability was tested by broth and filter mating assays using *E. coli* K12 strain BM21R (resistant to nalidixic acid and rifampin, positive for lactose fermentation, and free of plasmids) as recipient at a 1:2 donor: recipient ratio. Transconjugants were selected on Luria-Bertani agar plates containing cefotaxime (1 mg/L) and rifampin (100 mg/L) and incubated at 37°C for 24–48 h. Transformation was performed for a subset of isolates by using conditions reported (*3*). Chromosomal or plasmid location of $bla_{CTX\text{-}M\text{-}15}$ genes was assessed by hybridization of I-*Ceu*I–digested genomic DNA with $bla_{CTX\text{-}M\text{-}15}$ and 16S rDNA probes and electrophoresis (5–25 s for 23 h and 60–120 s for 10 h at 14°C and 6 V/cm^2) (*25*). Transfer and hybridization were performed by using standard procedures. Labeling and detection were conducted by using enhanced chemiluminescence (Amersham Life Sciences, Uppsala, Sweden) following manufacturer's instructions.

Plasmid Characterization

Plasmid DNA was obtained by using different midiprep plasmid purification kits (QIAGEN, Hilden, Germany, and Marlingen Biosciences, Ijamsville, MD, USA). Plasmids were classified according to their incompatibility group by a PCR-based replicon-typing scheme (*29*). Determination of plasmid size and confirmation of replicon content was established for transconjugants (or wild-type strains in the absence of transfer) by hybridization of S1 nuclease–digested genomic DNA with probes specific for $bla_{CTX\text{-}M\text{-}15}$ and for different F replicons (FII, FIA, FIB), which were obtained by PCR as described (*19*). Relationships among plasmids were determined by comparison of *Eco*RI and *Hpa*I digested DNA patterns and comparison of repFII sequences. Genescan software (Applied Biosystems, Foster City, CA, USA) was used for collection of gel images. Data of a subset of representative patterns were exported into Fingerprinting II Informatix version 3.0 software (Bio-Rad Laboratories, Hercules, CA, USA) for further interpretation. Cluster analysis was conducted by using the unweighted pair group method with arithmetic averages (optimization 0.5%, tolerance 1.00%).

Presence of genes previously associated with plasmids encoding CTX-M-15 as $bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1}$, and *aac(6')-Ib-cr* was screened by PCR by using primers $bla_{OXA\text{-}1}$ (oxa1 FW: 5′-TTT TCT GTT GTT TGG GTT TT-3′ and oxa1 RV: 5′-TTT CTT GGC TTT TAT GCT TG-3′), $bla_{TEM\text{-}1}$ (TEM-F: 5′-ATG AGT ATT CAA CAT TTC CG-3′ and TEM-R: 5′-CTG ACA GTT ACC AAT GCT TA-3′), and *aac(6')-Ib-cr* (aac-cr-F: 5′-TTG CGA TGC TCT ATG AGT GG-3′ and aac-cr-R: 5′-GCG TGT TCG CTC GAA TGC C-3′) (*11*,*19*,*30*). Additional sequencing was necessary to identify the corresponding genes.

Results

Epidemiologic Background

Most CTX-M-15–producing *E. coli* isolates belonged to phylogroups B2 (50%) and D (25%), which are known

to be associated with the hospital setting and extraintestinal pathogenic *E. coli*. Phylogroups A (18%) and B1 (7%), which are associated with animal or human commensal strains, were less frequently represented. All isolates of phylogroups B2, A, and D corresponded to subgroups $B2_3$, A_1, and D_1, respectively, which are the most common ones within each phylogenetic group (*31*). The 43 clinical isolates were classified into 32 PFGE types ($B2_3$, 13; D_1, 10; A_1, 6; and B1, 3). Among $B2_3$ strains, 10 PFGE types (18 isolates from France, Canada, Spain, Portugal, Kuwait, and Switzerland) were possibly related according to criteria of Tenover et al. (*32*) (difference ≤6 bands, ≥80% similarity) and were assigned to the sequence type (ST) ST131. The 4 unrelated B2 strains were classified within ST695 (1 from France), ST28 (1 from Switzerland), ST354 (1 from Portugal and Spain) and ST405 (1 from Portugal). All isolates of phylogroup D_1 were clonally unrelated by PFGE (difference ≥6 bands), although MLST studies indicated that 4 PFGE types (5 isolates) from Kuwait, Switzerland, and Spain corresponded to ST405. The *fumC* sequences of the remaining 6 *E. coli* D strains were highly diverse (alleles 4, 13, 26, 88, and 132). None of the strains had the C288T single nucleotide polymorphism specific for *E. coli* strain CgA (*28*). All 3 B1 isolates were found in France. Among B2 *E. coli* isolates, all but 4 were isolated from urine and all but 2 belonged to ST131. These strains correspond to 2 isolates recovered from wounds and identified as ST28 and ST354 and 2 ST131 isolates from respiratory and fecal samples, respectively.

CTX-M-15 clinical strains were considered resistant to different antimicrobial drugs: amoxicillin-clavulanate (98%), tobramycin (89%), kanamycin (87%), tetracycline (84%), gentamicin (82%), nalidixic acid (74%), streptomycin (68%), sulfonamides (61%), ciprofloxacin (61%), trimethoprim (58%), chloramphenicol (21%), nitrofurantoin (12%), and amikacin (11%). All CTX-M-15 transconjugants expressed resistance to aminoglycosides, tetracycline, or trimethoprim. All but 2 strains contained bla_{OXA-1} and *aac(6')-Ib-cr*; 1 contained only *aac(6')-Ib-cr*, and 1 contained bla_{OXA-1} and *aacA4*, which confers reduced susceptibility to amikacin and kanamycin.

Location and Transferability of $bla_{CTX-M-15}$

The $bla_{CTX-M-15}$ gene was located on plasmids in all but 6 strains and was positively transferred by conjugation or transformation in 37% of the strains tested. In 8 clinical isolates corresponding to 7 PFGE types, the probe for $bla_{CTX-M-15}$ hybridized in chromosomal bands (2 belonging to $B2_3$ ST131, 2 to D_1, 1 to D_1 ST405, and 1 to A_1). In 2 other strains, the $bla_{CTX-M-15}$ probe hybridized both with plasmid and chromosomal bands (1 strain from D ST405 and 1 from phylogroup B1).

Plasmids Encoding CTX-M-15

Plasmids positive for the $bla_{CTX-M-15}$ gene showed variable sizes (85–160 kb), belonged to the narrow host range incompatibility group IncF, and had replicon FII alone or in association with the FIA or FIB replicons (online Appendix Table, available from www.cdc.gov/EID/content/14/2/195-appT.htm). Many restriction fragment length polymorphism (RFLP) patterns were observed, with overrepresentation of 3 profiles corresponding to 3 plasmids arbitrarily designated as plasmid A (85 kb), plasmid B (120 kb), and plasmid C (85 kb). Plasmid A, which was isolated from B2 *E. coli* strains from 4 countries (India, France, Portugal, and Spain), was associated with different STs (ST131, ST354, or ST405). Plasmid C was also detected in clonally unrelated *E. coli* of phylogroups B2 and D from Switzerland, Canada and France. Plasmid B, which was only associated with *E. coli* ST131, was widely disseminated in all countries studied. Sequence analysis of the replicons showed 4 repFII types: repFII(1), which was identical to that of plasmids R100, NR1, or pC15–1a, and was the most represented and identified in 23 plasmids; repFII(2), which had 99%–100% homology with plasmid pRSB107 (GenBank accession no. AJ851089), was identified in 6 plasmids; and repFII(3) and repFII(4), which were detected in 2 and 7 plasmids, respectively, and showed >93% homology with repFII(1). All repFIA and repFIB sequences were 99% and 100% homologous, respectively, with that of pRSB107 (GenBank accession no. AJ851089).

Computer analysis of representative RFLP patterns and repFII sequences grouped CTX-M-15 plasmids within 3 major clusters with similarity >70%. Cluster I comprises most plasmids, including plasmids A and B, most containing repFII(1) and showing variable replicon content. Cluster II comprised only plasmid C derivatives showing slightly different repFII sequences, and cluster III included 2 plasmids carrying repFII(2), FIA, and FIB replicons (Figure).

In the 8 strains with chromosomal location of $bla_{CTX-M-15}$, repFII plasmids were identified but these plasmids were negative for the $bla_{CTX-M-15}$ gene. Several strains that were also positive for additional plasmids and negative for the $bla_{CTX-M-15}$ gene were assigned to different incompatibility groups or were untypeable by the PCR-based replicon typing scheme used.

Discussion

Our study indicates that current worldwide spread of the $bla_{CTX-M-15}$ gene is driven mainly by 2 epidemic *E. coli* strains belonging to phylogroups B2 (ST131) and D (ST405) and by its location on IncF plasmids harboring multiple antimicrobial drug–resistance determinants, including the recently described *aac(6')-Ib-cr* gene. The presence of $bla_{CTX-M-15}$ has previously been associated with *E. coli* strains of phylogroups B2 and D, and in some instances, with

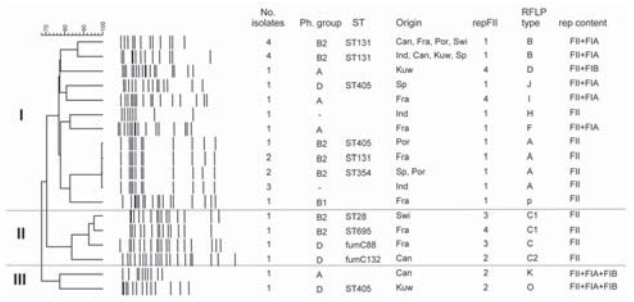

Figure. Computer analysis of a subset of representative *Hpa*I restriction profiles of IncF CTX-M-15 plasmids from *Escherichia coli* isolates in the Appendix Table. Cluster analysis was done by using Fingerprinting II in Informatix software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA) and applying the unweighted pair group method with arithmetic averages (optimization 0.5%, tolerance 1.00%). Ph, phylogenetic; ST, sequence type; RFLP, restriction fragment length polymorphism; Can, Canada; Fra, France; Por, Portugal; Swi, Switzerland; Ind, India; Kuw, Kuwait; Sp, Spain.

specific PFGE types (*9–12*,*16*). We detected an emerging and globally disseminated CTX-M-15 phylogroup B2 *E. coli* strain corresponding to the ST131 that was responsible for clonal outbreaks in Canada, France, Spain, and Portugal (*11*,*14*,*16*,*23*). Other CTX-M-15 B2 strains belong to clonal complexes ST695, ST405, ST354, or ST28, which have previously been detected in different geographic areas among isolates that do not express CTX-M-15 (online Appendix Figure, available from www.cdc.gov/EID/content/14/2/195-appG.htm).

Globally disseminated *E. coli* strains associated with acute, uncomplicated, community-acquired cystitis and pyelonephritis, designated in community patients as clone CgA (ST69), have only been occasionally associated with CTX-M-15 production in Canada (*16*,*27*,*28*). Although the isolates in our study do not belong to clone CgA, they were isolated mainly from urine samples, and an association of ST131 *E. coli* isolates with urinary tract infections might be inferred. Although most CTX-M-15 isolates studied were recovered from hospitalized patients, these microorganisms are now widely spread in the community setting, including long-term care facilities in the countries from which isolates included in this study originated (*2,5,14,33*). Our study has increased knowledge of the number of epidemic *E. coli* clonal complexes causing urinary tract infections.

All plasmids carrying $bla_{\text{CTX-M-15}}$ included in this study corresponded to incompatibility group F, and all had the FII replicon, which was assorted mainly in multireplicon plasmids with additional replicons of the FIA and FIB types. Association of the $bla_{\text{CTX-M-15}}$ gene with IncFII replicons has been described in studies conducted in Canada, France, Spain, and the United Kingdom (*5*,*7*,*8*,*17*,*19*). Although we observed intercontinental dissemination of 3 major IncFII plasmid scaffolds (A, B, and C) carrying $bla_{\text{CTX-M-15}}$, similarity >70% among all variants studied and presence of genes also found in pC15–1a, a CTX-M-15 plasmid (GenBank accession no. AY458016) that has a 28.4-kb multidrug resistance region containing $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, the *aac(6′)-Ib-cr* gene (aminoglycoside 6′-N-acetyltransferase type Ib-cr variant responsible for reduced susceptibility to both aminoglycosides and certain fluoroquinolones), and genetic determinants coding for resistance to tetracycline and aminoglycosides (*5*,*30*), suggest a common origin or a common particular plasmid scaffold involved in the dissemination of CTX-M-15.

Because IncF plasmids are a heterogeneous and largely diffused family of plasmids in *E. coli*, they could acquire the $bla_{\text{CTX-M-15}}$ gene. IncF plasmids negative for the $bla_{\text{CTX-M-15}}$ gene in strains with this gene at a chromosomal location also suggest dynamic horizontal exchanges between the chromosome and resident plasmids. Extensive recombination events among IncF plasmids are frequent and may have contributed to their apparent high diversity (variable rep content, plasmid size, transferability, antimicrobial drug–resistance genes), driving their evolution and enabling them to persist in diverse *E. coli* populations (*34,35*). Such recombination events among plasmids of the same incompatibility group within the same cell occur frequently (*34,35*). This hypothesis is supported by the results of Lavollay et al. (*17*), who described mosaicism in a CTX-M-15 plasmid isolated in France that contained genes from 2 different IncFII plasmids, pC15–1a and pRSB107 (from IncFII plasmids first isolated from persons in Canada and activated sludge bacteria from a wastewater treatment plant in Germany, respectively) (*5*,*36*).

Spread and maintenance of conjugative plasmids across bacterial populations have been intensively studied from a theoretical point of view, but data from natural populations are scarce (*34*,*37*,*38*). Recovery of related plasmids from clonally unrelated B2 strains might reflect efficient transfer of these elements among different B2 *E. coli* populations. Sharing the same environment, successive immigrant B2 strains might sweep through the population, enabling plasmid hitchhiking at a high frequency in each selective sweep. However, we lack detailed information on the specificity and stability of different plasmid groups in specific hosts. An evolutionary convergent relationship among B2 genetic background and IncFII plasmids cannot be ruled out and should be studied because it might explain successful dissemination of CTX-M-15 plasmids within this *E. coli* lineage. In addition, our study is one of the few that have identified bla_{ESBL} genes in the chromosome, which might respond either to plasmid integration or transposition driven by IS*Ecp1* located upstream from the $bla_{\text{CTX-M-15}}$ gene (*25*,*39*,*40*).

In conclusion, worldwide dissemination of $bla_{CTX\text{-}M\text{-}15}$ is driven by B2 or D *E. coli* clones associated mainly with urinary tract infections or IncFII plasmids containing a multiple antimicrobial drug–resistance platform that contributes to spread of CTX-M-15. Further studies to test the stability/variability and fitness of particular plasmids among different bacterial hosts will be relevant in developing additional strategies to control dissemination of antimicrobial drug resistance.

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Severe *Streptococcus pyogenes* Infections, United Kingdom, 2003–2004

Theresa L. Lamagni, MSc*; Shona Neal*; Catherine Keshishian, MSc*; Neelam Alhaddad, MSc*; Robert George, MD*; Georgia Duckworth*; Jaana Vuopio-Varkila†; and Androulla Efstratiou, PhD*

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After this activity the reader will be able to:

- Describe the geographic distribution of cases of *Streptococcus pyogenes* infection.
- Describe demographic profile of patients with *S. pyogenes* infection.
- Describe seasonal patterns of *S. pyogenes* infection.
- Describe the clinical presentation of patients with *S. pyogenes* infection.
- List 4 risk factors for *S. pyogenes* infection.
- List 4 potential public health actions for minimizing the effects of *S. pyogenes* infection.

Severe *Streptococcus pyogenes* Infections, United Kingdom, 2003–2004

Theresa L. Lamagni,* Shona Neal,* Catherine Keshishian,* Neelam Alhaddad,* Robert George,* Georgia Duckworth,* Jaana Vuopio-Varkila,† and Androulla Efstratiou*

As part of a Europe-wide initiative to explore current epidemiologic patterns of severe disease caused by *Streptococcus pyogenes*, the United Kingdom undertook enhanced population-based surveillance during 2003–2004. A total of 3,775 confirmed cases of severe *S. pyogenes* infection were identified over 2 years, 3.33/100,000 population, substantially more than previously estimated. Skin/soft tissue infections were the most common manifestation (42%), followed by respiratory tract infections (17%). Injection drug use was identified as a risk factor for 20% of case-patients. One in 5 infected case-patients died within 7 days of diagnosis; the highest mortality rate was for cases of necrotizing fasciitis (34%). Nonsteroidal antiinflammatory drugs, alcoholism, young age, and infection with *emm*/M3 types were independently associated with increased risk for streptococcal toxic shock syndrome. Understanding the pattern of these diseases and predictors of poor patient outcome will help with identification and assessment of the potential effect of targeted interventions.

Diseases caused by the Lancefield group A streptococcus *Streptococcus pyogenes* are among the most varied in terms of clinical spectra and severity, ranging from the ubiquitous pharyngitis to rarer life-threatening manifestations such as necrotizing fasciitis. Interest in these diseases was renewed after the United States and several countries in Europe reported increasing numbers of cases of invasive *S. pyogenes* disease during and since the 1980s (*1*). These apparent changes triggered several rapid global initiatives, coordinated by a World Health Organization working group, including review of microbiologic diagnostic methods and commencement of enhanced surveillance in several countries during the mid-1990s (*2*).

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In light of these changes, a cohesive network of 11 countries was formed in September 2002 to gain insight into the epidemiology of severe *S. pyogenes* disease across Europe. This network was funded by the European Union Fifth Framework Programme (*3*). To meet the Strep-EURO program objectives, the United Kingdom and other countries established population-based enhanced surveillance of severe *S. pyogenes* disease. Surveillance was undertaken to obtain accurate and comparable measures of overall and disease-specific incidence among participants and to compare demographic, risk factor, and clinical profiles of case-patients between countries, as well as microbiologic characteristics of *S. pyogenes* isolates collected.

Methods

In accordance with the program objectives, the Public Health Laboratory Service (now part of the Health Protection Agency) initiated enhanced surveillance of severe *S. pyogenes* disease from January 1, 2003, through December 31, 2004. Cases were defined according to the US definition (*S. pyogenes* isolated from a sterile site) (*4*). Also included were cases in which *S. pyogenes* was isolated from a nonsterile site in combination with probable streptococcal toxic shock syndrome (STSS) or another severe manifestation (pneumonia, necrotizing fasciitis, puerperal sepsis, meningitis, or septic arthritis). STSS was defined according to US specifications that differentiate between confirmed and probable cases on the basis of recovery of a sterile or nonsterile site isolate, respectively (*4*).

To maximize case ascertainment, cases were identified from 2 sources: isolate referrals to the national reference laboratory (Streptococcus and Diphtheria Reference Unit [SDRU]) and surveillance reports made to the Communicable Disease Surveillance Centre (CDSC) (*5*). SDRU pro-

vides reference microbiology services to the United Kingdom; CDSC surveillance covers England, Wales, Northern Ireland, the Channel Islands, and the Isle of Man. Cases identified from each source were reconciled with each other by using automated techniques to match records on the basis of personal identifiers (date of birth, sex, hospital number, National Health Service number, SOUNDEX-coded surname) and geographic location, followed by loose matching and manual checking to allow records to differ slightly on any given matching parameter. Referring laboratories were sent a study questionnaire to obtain further information on the demographic profile of the patient, disease manifestations, markers of clinical severity, outcome, and possible sources of infection (*6*). Ethnicity of patients was sought and classified according to census groupings for rate calculation. Where an isolate had not been received by SDRU, this isolate was also requested.

Group A streptococcal isolates referred to SDRU were characterized according to their M protein by using conventional serologic and *emm* gene typing (7,8). Antimicrobial drug–susceptibility testing was conducted by referral laboratories according to local standard operating procedures.

Responses to completed questionnaires were entered and stored in a custom-made Access (Microsoft, Redmond, WA, USA) database. All reports were checked to ensure they met the case definition. Repeat episodes were defined as those occurring in the same patient >30 days after the initial episode; reports received within 30 days were considered part of the same episode. Data were extracted for statistical analysis into STATA statistical software release 8.2 (Stata Corporation, College Station, TX, USA). Descriptive statistics were undertaken on confirmed cases with χ^2 and *t* tests used to test statistical significance of differences between subgroups. Incidence rates were calculated by using midyear resident population estimates for the respective years, age groups, sexes, and regional populations, with exact 95% confidence intervals (CIs) calculated according to the Poisson distribution. The 2001 census data were used as denominators for calculating rates according to ethnic group. All denominators were obtained from the Office for National Statistics. Stepwise unconditional logistic regression analysis was conducted to examine the independence of explanatory variables and development of STSS; the likelihood ratio test was used to evaluate significance of explanatory variables within each model.

All analyses were made on data from the United Kingdom, Channel Islands, and Isle of Man, except for estimated rates of infection, which were calculated for the areas with dual reporting (England, Wales, Northern Ireland, Channel Islands, and Isle of Man). The last 2 areas were omitted for age-, sex-, and ethnicity-specific rate calculations because of unavailability of these population denominators.

Results

Overview of Surveillance Results

From January 1, 2003, through December 31, 2004, a total of 3,821 cases of severe *S. pyogenes* disease meeting the case definition were reported from laboratories across the United Kingdom, Channel Islands, and Isle of Man. Of these cases, 21% were identified from isolate referrals only, without a corresponding surveillance report. Among the 3,821 reports were 46 repeat episodes, 5 of which were third episodes. Excluding repeat episodes, severe *S. pyogenes* disease was diagnosed for 3,775 patients in the United Kingdom, Channel Islands, and Isle of Man in 2003 and 2004.

S. pyogenes was isolated from a sterile site from 3,742 (99%) case-patients, primarily from blood culture (89%, 3,352). Thirty-three cases without sterile site isolates were included on the basis of $\geq$1 of the following clinical indicators: probable toxic shock syndrome (13 cases), necrotizing fasciitis (15), pneumonia (4), and puerperal sepsis (3).

Questionnaires were received for 2,647 (70%) of 3,775 case-patients. Information available for case-patients for whom questionnaires were or were not returned indicated their similarity in terms of age (median age 48 and 45 years, respectively), sex (54% male for both), and strain characteristics (*emm*/M type and erythromycin resistance), although a slightly higher proportion of case-patients for whom a questionnaire was returned had disease onset in December–April (53% vs. 48%; χ^2 6.37, degrees of freedom [df] 1, p = 0.012).

Geographic Distribution of Cases

In 2003 and 2004 combined, the overall rate of severe *S. pyogenes* infections was 3.33/100,000 population for England, Wales, Northern Ireland, Channel Islands, and Isle of Man. Variations were seen across these countries, with report rates higher in England (3,413 cases, 3.41/100,000) than Wales (153 cases, 2.60/100,000, rate ratio [RR] 1.32, 95% CI 1.12–1.55) or Northern Ireland (72 cases, 2.11/100,000, RR 1.62, 95% CI 1.28–2.04) but not significantly higher than rates in the Channel Islands and Isle of Man (10 cases, 1.98/100,000, RR 1.55, 95% CI 0.83–2.88). Substantial variations were also apparent between the English regions, with rates higher in Yorkshire and Humber (4.92/100,000) than in any other English region: East Midlands (3.25), East of England (2.98), London (2.75), North East (3.66), North West (3.70), South East (2.79), South West (3.92), and West Midlands (3.51). Report rates decreased in 2004 (1,718 cases, 3.12/100,000, RR 0.89, 95% CI 0.83–0.95) compared with 2003 (1,930 cases, 3.53/100,000); decreases in Yorkshire and Humber, and London accounted for 85% of this decrease.

Demographic Profile of Case-Patients

Severe *S. pyogenes* infection reports were highly concentrated in elderly persons (≥75 years of age, 10.67/100,000) and the young (<1 years of age, 9.70/100,000) (Figure 1). Rates for male patients were 22% higher than for female patients (3.65/100,000 vs. 2.98/100,000, RR 1.22; 95% CI 1.14–1.30); more male case-patients were found across all age groups, in particular, young adults (15–44 years of age), for whom rates were 61% higher for male than female patients (3.44/100,000 vs. 2.14/100,000, RR 1.61, 95% CI 1.43–1.80). Of 1,822 case-patients whose ethnicity was recorded, 1,727 (95%) were white, 58 (3%) from the Indian subcontinent, and 21 (1%) black African or Caribbean. Rates of severe *S. pyogenes* disease observed were significantly higher for whites (3.29) than for those of Indian subcontinent (2.46/100,000, RR 1.34, 95% CI 1.02–1.74) or black African or Caribbean origin (1.84/100,000, RR 1.79, 95% CI 1.16–2.75).

Seasonal Patterns of Infection

Marked seasonal peaks of severe *S. pyogenes* infection were observed in both years. Cases gradually increased from the end of October and first peaked near the end of January (2nd week of 2003, 51 cases; 4th week of 2004, 60 cases) before peaking again (higher) toward the end of March (12th week of 2003, 62 cases; 14th week of 2004, 64 cases) (Figure 2).

Clinical Manifestations of Cases

Clinical information was reported on the study questionnaire for 2,611 (69%) severe cases of *S. pyogenes* infection (Table 1). Approximately one fifth of patients were bacteremic but had no defined focus for infection. Of the remainder, skin/soft tissue was the most common focus of infection (42%, 1,099). Cellulitis was the most common manifestation, diagnosed for 881 (34%) patients; necrotizing fasciitis was diagnosed for 136 (5%) patients. Necrotizing fasciitis was rarely reported for pediatric case-patients (<16 years of age, 1%); most (64%, 87/136) cases occurred in persons 16–60 years of age. The respiratory tract was the next most commonly affected system; 434 (17%) patients showed clinical signs of upper or lower respiratory tract infection. Pneumonia was diagnosed for 309 (12%) case-patients.

Confirmed STSS developed in 196 (8%) patients who had a diagnosis of severe *S. pyogenes* infection (Table 2); 28% of these patients had necrotizing fasciitis. STSS developed in 40% (55/136) of patients with necrotizing fasciitis compared with 6% of other patients (χ^2 224.14, df 1, p<0.001). Use of nonsteroidal antiinflammatory drugs was positively associated with development of STSS (22% vs. 8%, χ^2 13.71, df 1, p<0.001).

Multivariable analysis of patient, clinical, and microbiologic factors associated with development of STSS identified age to be a strong predictor for STSS; risk for STSS was 5-fold greater for persons 15–44 years of age (odds ratio [OR] 5.42, 95% CI 2.22–13.23, p<0.001) than for the reference group (children <15 years of age). Persons 45–64 years of age had a 5-fold increased risk for STSS (OR 5.20, 95% CI 2.12–12.74, p<0.001). Patients >65 years of age had no increased risk for STSS compared with the pediatric reference group. Regardless of age, patients with necrotizing fasciitis had a 7-fold increased risk for STSS (OR 6.87, 95% CI 4.25–11.09, p<0.001).

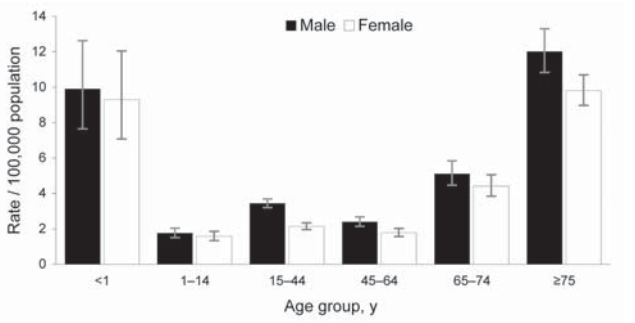

Figure 1. Age- and sex-specific annual incidence of severe *Streptococcus pyogenes* infection in England, Wales, and Northern Ireland, 2003–2004. Error bars show 95% confidence intervals.

Four risk factors (alcoholism, injection drug use, malignancy, and use of nonsteroidal antiinflammatory drugs) were independently associated with development of STSS. Patients who used nonsteroidal antiinflammatory drugs had a 3-fold increased risk for STSS (OR 3.00, 95% CI 1.30–6.93, p = 0.01). Alcoholism was associated with a 2-fold increased risk for STSS (OR 2.52, 95% CI 1.27–5.03, p = 0.008). Conversely, patients with malignancies had a much lower risk for STSS (OR 0.34, 95% CI 0.12–0.96, p = 0.042), as did injection drug users (OR 0.23, 95% CI 0.10–0.56, p = 0.001). Patients infected with an *emm*/M3 type, which was the only strain associated with STSS, had a 3-fold increased risk for STSS compared with patients infected with the reference group (*emm*/R28) (OR 3.20, 95% CI 1.35–7.58, p = 0.008).

Figure 2. Seasonal trends in reports of severe *Streptococcus pyogenes* infection in the United Kingdom, 2003–2004. Moving average (6 wk) is the average count for the previous 6 weeks.

Table 1. Clinical manifestations of severe *Streptococcus pyogenes* infection, United Kingdom, 2003–2004

Manifestation	All case-patients, no. (%)*	Children <16 y of age, no. (%)	Adults 16–60 y of age, no. (%)	Adults >60 y of age, no. (%)
Skin/soft tissue infection	1,099 (42)	85 (27)	479 (39)	531 (50)
Cellulitis	881 (34)	59 (19)	362 (30)	457 (43)
Necrotizing fasciitis	136 (5)	4 (1)	87 (7)	45 (4)
Abscess	134 (5)	6 (2)	112 (9)	16 (2)
Erysipelas	24 (1)	2 (1)	9 (1)	13 (1)
Bacteremia with no defined focus	558 (21)	77 (24)	228 (19)	252 (24)
Respiratory tract infection	434 (17)	66 (21)	187 (15)	181 (17)
Pneumonia	309 (12)	27 (8)	139 (11)	143 (13)
Other lower respiratory tract infection	62 (2)	6 (2)	26 (2)	30 (3)
Pharyngitis/tonsillitis	51 (2)	26 (8)	16 (1)	9 (1)
Ear infection	22 (1)	8 (3)	9 (1)	5 (<1)
Epiglottitis	17 (1)	2 (1)	10 (1)	5 (<1)
Sinusitis	6 (<1)	4 (1)	0	2 (<1)
Septic arthritis	220 (8)	40 (13)	114 (9)	66 (6)
Puerperal sepsis	58 (2)	0	58 (5)	0
Acute abdominal infection†	49 (2)	5 (2)	31 (3)	12 (1)
Cardiac infection‡	48 (2)	2 (1)	36 (3)	10 (1)
Meningitis	37 (1)	18 (6)	12 (1)	7 (1)
Total	2,611 (100)	318 (100)	1,225 (100)	1,064 (100)

*Includes patients without age information.
†Includes 14 patients with peritonitis, 1 with appendicitis, and 1 with pancreatitis.
‡Includes 42 patients with endocarditis, 4 with pericarditis, 1 with myocarditis, 1 with myocarditis and pericarditis.

Overall, 413 (19%) patients for whom *S. pyogenes* infection was the main underlying or contributory cause of death died within 7 days of initial microbiologic diagnosis. Necrotizing fasciitis was the most severe clinical manifestation for patients, according to specified markers; patients with this condition were most likely to be admitted to an intensive care unit (ICU) (77%) or to die within 7 days of diagnosis (34%). However, ICU admission (20%) and surgical intervention (24%) were not uncommon among other patients. Case-fatality rates were also high for patients with pneumonia, 32% of whom died within 7 days. Of the 58 young women in whom puerperal sepsis developed, 2 died. Development of STSS was strongly linked to risk for death; 84 (45%) of 185 patients with STSS died from their infection compared with 329 (16%) of 2,007 without STSS.

Patient Risk Factors

Information on risk factors was available for 61% of case-patients with severe *S. pyogenes* infection. Of these, lesions or wounds to the skin were reported for 31% (719). Skin is the most likely portal of entry recorded overall, especially among persons >60 years of age, 40% of whom had skin lesions. Information on the nature of these lesions was available for 617 case-patients. The 2 most common types were traumatic lesions (188) and chronic wounds (161). Traumatic lesions were most common among young adults (16–60 years of age, 9%); chronic wounds were most common among elderly persons (14%). Less common types of wounds were recorded that included insect bites (21 cases, 0.9%) and animal-associated traumatic wounds (cat scratches and dog and human bites, 8 cases, 0.3%).

Of young adults with severe *S. pyogenes* infections, 459 (40%) were injection drug users (20% of case-patients of all ages). Other conditions commonly reported that could have predisposed persons to infection included malignancies (161) and diabetes (158), each noted for 7% of cases overall and 11% and 13%, respectively, among elderly persons. Nine percent (204) of infections were associated with healthcare, mostly postsurgical infections (118). Among pediatric case-patients (<16 years of age), varicella was the next most common predisposing factor noted after skin lesions, reported for 41 (14%) children. Overall, 566 (25%) case-patients did not have any particular predisposition, or risk for severe *S. pyogenes* infection on the basis of the common factors outlined (Table 3) or any others considered pertinent by the reporting clinician. Among pediatric case-patients, this proportion increased to 46% (132).

Discussion

As part of a wider European initiative to improve our understanding of the epidemiology of severe *S. pyogenes* infections, the United Kingdom has amassed one of the largest collections of such cases recorded. The >3,700 cases diagnosed in 2003–2004 resulted in a rate of 3.33/100,000 population for England, Wales, Northern Ireland, the Channel Islands, and Isle of Man. This rate was similar to rates reported for other European countries and the United States in the early 2000s (*1*,*9*,*10*), although lower than some estimates from Canada (*11*–*13*). In the 2-year study period, the overall rate of severe *S. pyogenes* infections decreased from

Table 2. Clinical markers of severe *Streptococcus pyogenes* infection in the United Kingdom, 2003–2004*

Marker	All case-patients (n = 2,611), no. (%)	Bacteremia with no defined focus (n = 558), no. (%)	Pneumonia (n = 309), no. (%)	Cellulitis (n = 881), no. (%)	Septic arthritis (n = 220), no. (%)	Necrotizing fasciitis (n = 136), no. (%)	Puerperal sepsis (n = 58), no. (%)
Clinical severity (n = 2,611)							
Hypotensive shock	494 (19)	101 (18)	70 (23)	155 (18)	43 (20)	80 (59)	13 (22)
STSS (hypotension + ≥2 listed below)	196 (8)	28 (5)	27 (9)	63 (7)	18 (8)	55 (40)	3 (5)
Renal impairment	381 (15)	69 (12)	50 (16)	149 (17)	40 (18)	58 (43)	3 (5)
Respiratory distress	288 (11)	41 (7)	113 (37)	67 (8)	20 (9)	33 (24)	1 (2)
Erythematous rash	231 (9)	30 (5)	15 (5)	135 (15)	13 (6)	17 (13)	4 (7)
Soft-tissue necrosis	254 (10)	0	8 (3)	116 (13)	13 (6)	136 (100)	3 (5)
Liver abnormality	185 (7)	31 (6)	23 (7)	65 (7)	14 (6)	31 (23)	3 (5)
Disseminated intravascular coagulation	131 (5)	21 (4)	14 (5)	40 (5)	10 (5)	35 (26)	6 (10)
Admission to ICU (n = 2,292)	451 (20)	76 (16)	68 (24)	136 (17)	40 (21)	101 (77)	8 (16)
Surgical intervention (n = 1,885)	443 (24)	21 (6)	19 (8)	131 (20)	106 (63)	106 (87)	10 (24)
Death within 7 d (n = 2,192)	413 (19)	97 (22)	87 (32)	121 (16)	17 (9)	43 (34)	2 (4)

*STSS, streptococcal toxic shock syndrome; ICU, intensive care unit.

3.53/100,000 to 3.12/100,000. This overall decrease was largely caused by a substantial decrease in 2 regions, Yorkshire and Humber, and London. Use of multiple sources for case ascertainment was an improvement over previously used methods. Previous methods, which relied solely on voluntary laboratory reporting, would have yielded a rate of 2.65/100,000 during this period. As with any study dependent on participation of local reporters, this study may have missed additional diagnosed cases.

Rates of severe diseases associated with *S. pyogenes* were markedly higher in male patients than in female patients, an observation not consistently found in other countries (*12*,*14*) but commonly found among patients with bacteremic infections in the United Kingdom (*15*,*16*). We did not observe any increased rate of severe *S. pyogenes* infections in black patients of African or Caribbean origin, as was found in a large study in the United States (*17*). Estimated rates were lower for black patients than for Asian or white patients. Because our study relied on clinician and microbiologist reporting of ethnicity, the proportion of patients reported as white may have been overestimated as a result of assumptions made without confirmatory information.

Marked seasonal patterns in severe *S. pyogenes* disease were evident during the study period, with an initial peak in December–January, followed by a strong peak in March–April. Preliminary comparisons among Strep-EURO participants suggest similar patterns in other European countries (*18*). Why these diseases should peak in late winter and early spring is not known. Seasonal patterns of viral respiratory infections with respiratory syncytial virus and influenza virus, which could make patients vulnerable to invasive *S. pyogenes* infections, may play a role in early- to mid-winter *S. pyogenes* peaks but would not explain the main spring peak seen in the United Kingdom (Health Protection Agency, unpub. data).

Clinical information provided for these case-patients highlights the severity of these infections; 19% died within 7 days of the initial culture-positive specimen being obtained. This finding is consistent with overall case-fatality estimates during enhanced surveillance in the United Kingdom in 1994–1997 (25%) and estimates reported in other countries (*14*,*17*,*19*,*20*). However, only 1 of these studies defined a time frame for estimates or included the role of infection in the patient's death, as our study did. Case-fatality rates were particularly high for case-patients with necrotizing fasciitis, who accounted for only 5% of all cases but 10% of all deaths. Completion of the questionnaire could also have been biased in favor of more severe or interesting cases.

Among case-patients identified in this study, 12% had pneumonia, a value substantially higher than that noted by enhanced surveillance in the United Kingdom in 1994–1997 (5%) (*20*). However, our finding was consistent with those in studies in other countries (*11*,*13*,*17*,*19*,*21*,*22*). The case-fatality rate in our study (32% within 7 days of initial diagnosis) was substantially higher than that expected with community-acquired pneumonia (*23*).

STSS developed in 8% of the case-patients identified in our study. These patients had poor survival rates; 45% died within a week of initial diagnosis. STSS was most likely to develop in young adults, which is consistent with findings of a US study that reported a lower median age for STSS patients (*24*). Infection with an *emm*/M3 strain was

Table 3. Potential predisposing factors for severe *Streptococcus pyogenes* infection, United Kingdom, 2003–2004

Factor	All case-patients, no (%)*	Children <16 y of age, no. (%)	Adults 16–60 y of age, no. (%)	Adults >60 y of age, no. (%)
Skin lesion/wound	719 (31)	63 (22)	298 (26)	358 (40)
Trauma	188 (8)	13 (5)	99 (9)	76 (9)
Chronic wound	161 (7)	0	39 (3)	122 (14)
Surgery	118 (5)	10 (4)	48 (4)	60 (7)
Injection drug use	459 (20)	0	459 (40)	0
Healthcare-associated infection	204 (9)	15 (5)	79 (7)	109 (12)
Malignancy	161 (7)	11 (4)	49 (4)	100 (11)
Diabetes	158 (7)	3 (1)	40 (4)	115 (13)
Alcoholism	88 (4)	0	67 (6)	21 (2)
Recent childbirth	86 (4)	0	86 (8)	0
Steroid use	77 (3)	3 (1)	35 (3)	39 (4)
Contact with person with *S. pyogenes* infection†	57 (4)	7 (4)	34 (5)	16 (2)
Nonsteroidal antiinflammatory drug use	49 (2)	3 (1)	18 (2)	28 (3)
Varicella	47 (2)	41 (14)	4 (<1)	2 (<1)
Cardiovascular disease	45 (2)	3 (1)	9 (1)	33 (4)
Upper respiratory tract infection	39 (2)	9 (3)	24 (2)	6 (1)
Renal impairment	31 (1)	2 (1)	19 (2)	10 (1)
Other reported risk factor‡	112 (5)	30 (11)	41(4)	41 (5)
No risk factors reported	566 (25)	132 (46)	173 (15)	260 (29)
Total	2,305 (100)	284 (100)	1,135 (100)	884 (100)

*Includes patients without age information.
†Information available for 1,586 patients.
‡Noted for <30 patients.

associated with an increased risk for STSS. This finding is consistent with previous (unadjusted) findings from the United States (*17*) but different from findings from Canada, which only identified *emm*/M9, a strain uncommon in the United Kingdom, as associated with STSS (*13*). Alcoholism was associated with increased risk for STSS; this association was also found in other studies (*13*,*22*). However, risk for STSS was 3-fold greater for patients who were reported to have used nonsteroidal antiinflammatory drugs, despite adjustment for whether patients had necrotizing fasciitis, which is strongly associated with STSS. Because no data were collected about time, dose, indications for use, or which agent was taken, a causal link between use of nonsteroidal antiinflammatory drugs and STSS cannot be inferred from our findings. A confounding factor, such as delay in receiving appropriate treatment, which we did not adjust for in our analysis, could explain this finding. Patients who took nonsteroidal antiinflammatory drugs may have had early signs, such as extreme pain, which indicated a more severe infection. Nonetheless, this remains an interesting and potentially important observation in a controversial area where evidence supporting either immunologic impairment or augmentation caused by nonsteroidal antiinflammatory drugs remains unresolved (*25*). Other novel associations with STSS were made; e.g., STSS was less likely to develop in injection drug users or patients with malignancies than in other patients. These findings point to immunocompetence as a necessary mediator for development of STSS.

Questionnaires concerning possible predisposing factors have highlighted skin lesions as the most commonly identified potential source of infection, which is similar to findings in other countries (*18*,*22*). However, given the occult nature of *S. pyogenes* infections, as indicated by the high proportion (21%) of cases with no identified focus, several of these cases may have originated from respiratory tract colonization. This colonization could lead to transient bacteremia, which in turn seeded local tissue sites, possibly in the presence of local trauma.

In contrast to preliminary findings from other Strep-EURO participants (*18*), a substantial proportion of case-patients were injection drug users (20% overall and 40% among young adults). Regional breakdowns of risk factors were not undertaken in our analyses. However, regional differences in report rates are in part explainable by injection drug use–related cases, with highest overall rates in the Yorkshire and Humber region. This region had been identified as having a particular problem of severe *S. pyogenes* disease in injection drug users (*26*). Patterns of isolate referrals to the national reference laboratory over the past decade suggest an increase in severe *S. pyogenes* infection in injection drug users (*27*). The reasons behind this change remain unclear and warrant further investigation.

Most severe *S. pyogenes* cases in our study occurred sporadically in the community; only 9% were associated with healthcare interventions. One fourth of all case-patients and nearly 1 in 6 young adults had no particular risk factors identified. These findings highlight the likely eco-

nomic effect of these infections and the challenges in developing any effective prevention measure.

Analysis of information collected in this study has yielded some unique insights into these infections and has begun to provide an evidence base for mounting public health initiatives in the United Kingdom (*28*). High and rapidly ensuing mortality rates among these patients emphasize the need for early recognition and rapid treatment. Maintaining a high index of suspicion, especially where there are signs of possible necrotizing fasciitis, could clearly be lifesaving. Further analysis of *emm*/M type distribution, a key virulence factor inducing immunologic memory, will assist in assessment of the potential effect of vaccines currently under development. Changes in the epidemiology of severe *S. pyogenes* infections since the last period of enhanced surveillance in the mid-1990s underline the need for periodic monitoring to detect changes in disease manifestations, risk groups, and microbiologic characteristics to develop strategies for control and management of these infections.

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Effectiveness of Personal Protective Measures to Prevent Lyme Disease

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After the manufacture of Lyme vaccine was discontinued in 2002, strategies to prevent Lyme disease (LD) have focused on personal protective measures. Effectiveness of these measures has not been conclusively demonstrated. The aim of our case–control study was to assess the effectiveness of personal preventive measures in a highly disease-endemic area. Case-patients were persons with LD reported to Connecticut's Department of Public Health and classified as having definite, possible, or unlikely LD. Age-matched controls without LD were identified. Study participants were interviewed to assess the practice of preventive measures and to obtain information on occupational and recreational risk factors. Use of protective clothing was 40% effective; routine use of tick repellents on skin or clothing was 20% effective. Checking one's body for ticks and spraying property with acaricides were not effective. We concluded that use of protective clothing and of tick repellents (on skin or clothing) are effective in preventing LD.

Lyme disease (LD) is the most commonly reported tick-borne illness in the United States (*1*,*2*). A vaccine against the disease was licensed in 1998 for use in persons 15–70 years of age (*3*). However, because of poor sales, it was withdrawn from the market in 2002. Recommendations for preventing LD currently focus on personal protective measures and interventions to reduce abundance of vector ticks (*4*). Strategies include avoidance of tick-infested areas, use of protective clothing (i.e., wearing long-sleeved shirts and long pants, which decrease the area of exposed skin), routine checks of one's body for ticks, and the use of tick repellents on either the skin or clothing. Other strategies have targeted the environment and vertebrate hosts of deer ticks; however, such approaches are often impractical, and their effects on the incidence of LD are unknown. Finally, use of antimicrobial prophylaxis in selected persons who have been bitten by a deer tick may be effective (*5*).

Although personal protective measures are frequently recommended by medical providers, public health officials, and the lay press in areas where the disease is endemic, few data about their effectiveness exist. In 1992, Herrington et al. (*6*) found that self-perceived risk of acquiring LD and knowledge about the disease correlated with the use of personal protective measures; however, even in highly LD-endemic areas such as Connecticut, adherence to these personal protective measures varied greatly—only 79% of respondents routinely used tick repellents and 93% reported inspecting themselves for and removing ticks after being outdoors. In 2001, Phillips et al. found that 80% of the surveyed residents of Nantucket reported checking their bodies for ticks after potential tick exposures, 53% used protective clothing (such as wearing long pants and long-sleeved shirts), 34% reported routinely avoiding tick infested areas, and 11% reported routine use of tick repellents (*7*). These researchers did not find a difference in reported frequency of LD among those who did and those who did not report preventive behavior. Similarly, Orloski et al. did not find any statistically significant differences in use of protective measures between persons with LD and age-matched controls (*8*).

Although certain occupations, such as working outdoors in forestry or landscaping, have been suggested to increase the risk for LD, few studies document increased risk among such workers. Smith et al. evaluated outdoor workers by using questionnaires and serologic tests for antibody to *Borrelia burgdorferi* and found that workers with a history of outdoor employment were twice as likely to

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be seropositive as those without such a history; however, this difference was not statistically significant (*9*). A study conducted in California by Lane et al. found that outdoor occupations such as woodcutting were associated with an increased risk for LD (*10*).

We conducted a matched case–control study of persons 15–70 years of age residing in Connecticut. The purpose was to assess the effectiveness of Lyme vaccine and personal protective measures against LD. The study ceased soon after Lyme vaccine was withdrawn from the market.

Methods

Study Population

We conducted a matched case–control study from July 2000 to February 2003. Study participants were identified through an active surveillance system for LD initiated in 1991. Physicians in private practice and other health maintenance organizations throughout the state participated in the surveillance system. Staff of the State of Connecticut Department of Public Health (CT-DPH) contacted healthcare providers regularly to inquire about newly diagnosed cases of LD. Supplemental follow-up reporting forms were also sent to physicians when additional clinical information was needed. The study was approved by the Human Investigations Committee at the Yale University School of Medicine and the Institutional Review Boards of the CT-DPH and the Centers for Disease Control and Prevention (CDC).

Potential case-patients were persons 15–70 years of age (because Lyme vaccine was approved for persons in this age range) reported by their healthcare provider to CT-DPH as having LD in the period from January 2000 to February 2003. Study participants were enrolled prospectively (i.e., as each case was identified, case-patients and controls were recruited and enrolled). Letters of invitation were sent to potential case-patients after the reporting physician gave permission to contact the patient.

Controls were persons 15–70 years of age without LD who were matched to the case-patients by age (±5 years from case-patient's birth date) and who had a telephone. Controls were identified by using sequential-digit dialing, a technique that uses sequential digits from the end of the telephone number of the case-patient to contact potential controls (*11*). This technique also ensured that the case-patient and control resided in the same general geographic area. To maximize the likelihood of contacting control study participants, telephone calls were made on nonconsecutive days, including at least 1 weekend day (during daytime and evening hours). For each case-patient, we enrolled up to 2 matched controls. Controls were excluded if they had had LD 30 days before the diagnosis of LD in the matched case-patient (as stated either by them during the interview or as documented in their medical record), but neither case-patients nor controls were excluded if they had had LD in the more distant past, since a previous history of the disease was not a contraindication for the vaccine and LD can be acquired more than once (*12*).

Data Collection

Trained personnel obtained informed consent and interviewed both case-patients and controls by telephone with a standardized survey. For study participants <18 years of age, a parent or guardian was interviewed. Case-patient study participants were interviewed within the year of their Lyme diagnosis. Questions concerning demographics, occupational (forestry or landscaping) and recreational risk factors (camping or other outdoor activities), personal protective measures (specifically the use of tick repellents on the skin or clothing while outdoors; spraying one's property with acaricides; use of protective clothing such as long pants, long-sleeved shirts, and light-colored clothing; and checking one's body for ticks after being outdoors) to prevent LD were asked of all study participants; for case-patients, questions about clinical signs and symptoms were asked. The questions were phrased to discuss behavior of the case-patient before the diagnosis of LD; for control study participants, we asked about behavior before the date in which their matched case was diagnosed. For example, for a case-patient who received a diagnosis in July 2002, we asked him or her to respond to the questions on behavior before the LD diagnosis in July 2002: "Do you check your body routinely for ticks after outdoor exposure?" For the case-patient's matched control, we asked the same question, using the last 12 months before the diagnosis of the case as the reference period. Questions are shown in Table 1.

The medical records of case-patients and matched controls were reviewed. Information was recorded about receipt of Lyme vaccine (a case-patient was considered vaccinated if at least 1 dose of Lyme vaccine was received >30 days from the date of the LD diagnosis; for controls, we used the dates of the matched case-patient), previous medical history and, for case-patients, clinical and laboratory data at the time of diagnosis. Case-patients and controls were excluded from the analysis if their medical records could not be obtained.

Classification of LD

Case-patients were classified into 3 categories by 2 investigators, who were blinded to the case-patient's name, age, source of medical care, vaccination status, and use of personal protective measures; disagreements were resolved by discussion. The category of definite LD included study participants who met the surveillance case definition, i.e., erythema migrans (EM) measuring at least 5 cm in diame-

Table 1. Questions asked in the survey regarding risk factors and personal protective measures

Do you (your child) live in close proximity to grassy fields or heavily wooded areas?
Do you (your child) have an occupational exposure that puts you at risk for tick bites (such as working in landscaping, forestry, farming, or wild-life parks management)?
Do you (your child) engage in outdoor activities that put you at higher risk for tick bites (such as hiking, camping, gardening, hunting)?
Do you (your child) wear clothing to protect against ticks while outdoors, e.g., long pants, long-sleeved shirts, or light-colored clothing?
Do you (your child) routinely use tick repellents on the skin and/or clothing while outdoors?
Do you routinely spray acaricides against ticks on your property?
Do you (your child) routinely check for ticks on the body after being outdoors?
Do you have any pets at home?

ter documented by a physician, objective signs of early disseminated disease with a positive test result for antibodies against *B. burgdorferi* (if EM was not present), or objective signs of late disease with a positive test for antibodies against *B. burgdorferi* (the patient must have had antibodies measured by using the 2-tiered system recommended by CDC). The category of possible LD included those study participants who met most, but not all, of the criteria in the surveillance case definition, such as a case with EM that was either <5 cm in diameter or a size that was not documented. The third category included those study participants who were unlikely to have LD, i.e., they had only nonspecific symptoms, such as fatigue, and case-patients with objective findings but with negative serologic test results for LD. In addition, we classified all reported cases of LD according to the clinical stage of LD at the time of the diagnosis: early localized disease (single EM), early disseminated disease (multiple EM, early neurologic disease or cardiac disease), or late LD (arthritis or encephalomyelitis).

Statistical Analysis

Matched odds ratios (ORs), associated 95% confidence intervals (CIs), and their statistical significance were calculated by using SAS software (SAS Institute, Cary, NC, USA). Conditional logistic regression (EGRET Windows; CYTEL Corp., Cambridge, MA, USA) was used to adjust the matched ORs for the effects of potential confounding factors, including sex, use of other personal protective measures, receipt of Lyme vaccine, and race. Effectiveness was calculated as 1 = the matched OR; p values <0.05 were considered significant.

Results

We identified 1,436 age-eligible potential case-patients from January 2000 to February 2003. Thirty-two reported case-patients (2%) were not contacted because the reporting physician refused to permit it. Of the remaining 1,404 case-patients, we were unable to contact 340 (24%). Of the 1,064 who were contacted, 195 (18%) refused to participate (most refused to give consent to review their medical records). We enrolled and interviewed the remaining 869 potential case-patients (82%). Of those enrolled in the study, matched controls were not identified for 78 (9%), and data were incomplete for 82 (9%) (i.e., medical records could not be found). Data presented were part of a larger study of the effectiveness of Lyme vaccine. The study ended when the Lyme vaccine was withdrawn from the market and controls had not yet been obtained for all study participants.

Characteristics of the 709 case-patients in the study who had at least 1 matched control and the 1,128 matched controls are shown in Table 2. The age at the time of infection ranged from 15 to 70 years (median 48 years). Of the 709 cases (419 cases had 2 controls and 290 had 1 control), 467 (66%) were classified as definite cases of LD, 105 (15%) as possible cases, and 137 (19%) as unlikely to be LD. We found controls were more likely to be female; this difference could be explained by our method of identification and enrollment of controls (by telephone), if women were more likely to be at home at the time of our call. Similar proportions of case-patients and matched controls had received Lyme vaccine.

Tables 3 and 4 show risks factors for and use of personal protective measures against LD for case-patients, stratified by certainty of the diagnosis, and their matched controls. Adjustment for potential confounding variables such as gender, race, receipt of Lyme vaccine, and use of other personal protective measures was made with conditional logistic regression. Patients classified as having either definite or possible cases of LD (N = 572) were not significantly more likely to report risk factors for LD than their matched controls (N = 898). Definite and possible case-patients were less likely than controls to report using protective clothing outdoors (46% vs. 58%; adjusted OR 0.6, 95% CI 0.5–0.8; effectiveness 40%, p<0.0001) and to use tick repellents on their skin or clothing (29% vs. 34%, adjusted OR 0.8, 95% CI 0.6–1.0; effectiveness 20%, p = 0.05). Spraying acaricides routinely on one's property did not differ significantly for case-patients and matched controls. Estimates did not change significantly when only those cases classified as definite LD were analyzed (Table 3).

Recreational outdoor activities, such as hiking and camping (85% vs. 83%, p = 0.34), living near heavily wooded or grassy areas (95% vs. 94%, p = 0.18), and having pets at home (75% vs. 12%, p = 0.17), were not statistically significantly associated with LD in any of the

Table 2. Characteristics of Lyme disease study participants, Connecticut, July 2000 through February 2003

Characteristics	Case-patients (N = 709), no. (%)	Controls (N = 1,128), no. (%)	p value
Age, y			
Median	48	49	0.71
Mean	46	47	
Range	15–70	15–70	
Sex			
Female	376 (53)	715 (63)	<0.001
Male	333 (47)	413 (37)	
Race			
Caucasian	689 (97)	1094 (97)	0.66
African American	3	8	
Hispanic	4	6	
Other	13	14	
Underlying medical problems other than Lyme disease (e.g., diabetes, asthma)	298 (42)	508 (45)	0.19
Had Lyme disease*	110 (17)	143 (14)	0.095
Received Lyme vaccine†	44 (6)	73 (6)	0.82

*Having Lyme disease was defined as the following: for case-patients, having Lyme disease at a time other than the episode for which a case-patient was enrolled in the study; for controls, having Lyme disease at a time other than the focal time of disease for the case-patient.
†A study participant was considered vaccinated if he or she received at least 1 dose of Lyme vaccine.

groups. Only among groups in which the case-patient was classified as unlikely to have LD were patients significantly more likely than matched controls to have an occupational risk factor for LD such as forestry or landscaping (28% vs. 12%, adjusted OR 3.1, 95% CI 1.6–5.9, p = 0.0007). None of the personal protective measures was effective in groups classified as unlikely to have LD.

We also analyzed the data excluding all controls with a previous history of LD, and the results were virtually unchanged except that having an occupational exposure was a risk factor. When we excluded controls who had previously had LD from the analysis, case-patients were more likely to have an occupational exposure than were matched controls; this was true for definite cases of LD (60 [15%] of cases vs. 51 [9%] of controls; OR 1.5, 95% CI 0.9–2.4, p = 0.05), for definite cases and possible cases (78 [16%] of cases vs. 69 [10%] of controls; OR 1.5, 95% CI 1.03–2.2, p = 0.03), and for unlikely cases (34 [28%] of cases vs. 16 [9%] of controls; OR 3.6, 95% CI 1.8–7.7, p = 0.0006).

Discussion

This is the first study, to our knowledge, to demonstrate that any personal protective measure against LD is effective. Use of protective clothing while outdoors was 40% effective in strata with case-patients classified as having definite or possible LD. Of note, this strategy was not significantly effective in strata with case-patients classified as unlikely to have LD (not only were the differences between patients and controls in this group not statistically significant, but the magnitude of the effect was much diminished), which supports the validity of the results (*13*). The use of tick repellents on the skin or clothing while outdoors was also effective (effectiveness of 20%) for preventing LD.

By contrast, inspecting one's body for and removing ticks was not found to be an effective strategy to prevent LD nor was using acaricides on one's property. A potential limitation of the study is that use of the protective measures was based on self-report, so these practices could neither be confirmed nor quantified. Undoubtedly, there is variability in the practice of these personal protective measures that was not ascertained by the study. For example, some persons may only perform cursory tick checks, while others may engage in a more careful examination. Similarly, there may be variability in the application of tick repellents—in the amount and frequency of application and whether the repellents were applied to clothing, to exposed skin, or to both, as well as variations in repellents themselves. (Active ingredients such as DEET [N,N-diethyl-3-methyltoluamide], permethrin, or natural or herbal repellents and their concentrations may vary.) Whether rigorous practice of these protective measures might be protective is unknown. Furthermore, we did not assess for ecologic differences that may have played a role in the possibility for household acaricides to have been effective or for risk factors for the disease, such as apartment versus single family dwelling. The effectiveness of the use of acaricides may also possibly rely on routine use not only by the study participant but also by close neighbors.

We used sequential-digit technique, which ensures that controls are generated from the same general area. We did not analyze differences in locations within telephone exchanges; however, LD is considered to be endemic in the entire state of Connecticut, and no major differences would be expected within areas of a telephone exchange. Another potential limitation of our study is recall bias—study participants are more likely to remember things related to LD if they have had the disease itself.

Table 3. Personal protective measures and risk factors for Lyme disease, Connecticut, July 2000 through February 2003

Personal protective measures	Case-patients, no. (%)	Matched controls, no. (%)	Odds ratio* (95% CI)†	p value
Use of protective clothing while outdoors				
Definite	215 (46) N = 467	427 (59) N = 724	0.6 (0.5–0.7)	<0.0001
Definite and possible	265 (46) N = 572	524 (58) N = 898	0.6 (0.5–0.8)	<0.0001
Unlikely	72 (53) N = 137	121 (53) N = 230	0.9 (0.6- 1.3)	0.55
Use of tick repellents on skin or clothing while outdoors				
Definite	138 (30) N = 467	252 (35) N = 724	0.8 (0.6–0.9)	0.04
Definite and possible	168 (29) N = 570	303 (34) N = 890	0.8 (0.6–0.99)	0.0499
Unlikely	37 (27) N = 136	64 (28) N = 228	0.9 (0.6–1.5)	0.83
Spraying property with tick acaricides				
Definite	16 (7) N = 237	52 (11) N = 467	0.6 (0.3–1.1)	0.09
Definite and possible	19 (7) N = 285	62 (11) N = 557	0.6 (0.3–1.0)	0.06
Unlikely	3 (8) N = 36	16 (12) N = 133	0.7 (0.2–3.0)	0.61
Checking the body for ticks after exposure				
Definite	360 (77) N = 467	560 (77) N = 724	1.1 (0.8–1.4)	0.64
Definite and possible	443 (77) N = 572	703 (78) N = 898	1.0 (0.8–1.4)	0.81
Unlikely	107 (78) N = 137	181 (79) N = 230	0.9 (0.5–1.5)	0.61

*All estimates were adjusted for possible confounders (sex, race, receipt of Lyme vaccine, and use of other personal protective measures) with conditional logistic regression.
†CI, confidence interval.

The finding that occupational exposure did not appear to be a risk factor for cases classified as definite or possible LD suggests that most of the case-patients with true LD reported in Connecticut acquired their LD from periresidential or recreational exposure. However, when we excluded controls with a history of LD in the past, occupational exposure was a risk factor for the disease. The finding that case-patients classified as unlikely to have LD were more likely to have had occupational exposures than the corresponding controls is somewhat perplexing. Possibly persons who are in occupations that have been associated with LD and who are aware of their potential risk for the disease are more likely to seek medical attention for suspected LD symptoms, and their physicians may be more likely to report them as LD cases because of the perceived occupational risk. Such a diagnostic bias would tend to artificially elevate the prevalence of occupational exposure among these persons suspected to have LD who in fact probably do not have LD. It is also possible that occupational exposure is a true risk factor for the other conditions that are causing symptoms in these persons who are unlikely to have LD.

Public health strategies are important in the control of LD, an emerging infection with continually increasing incidence (*1*); nonetheless, the implementation and assessment of these strategies have proven to be challenging. Public health officials must take into account not only the effectiveness of the public health strategy, but also the level of engagement of those who are supposed to follow the recommendations (*14*). Although this study indicates that use of protective clothing and the use of tick repellents on the skin or clothing while outdoors are effective, clearly not all persons at risk follow these recommendations. Our results provide data to support recommendations that have been in place for many years. By no means should our results be taken as recommendations to cease personal protective measures that have been recommended by public health officials but that we found not to be effective. Although our study included adolescents, most study participants were adults; therefore, whether these findings apply to children, a group at high risk of acquiring LD, is not known.

Additional educational efforts about these practices, targeted at persons living in LD-endemic areas, may be beneficial. The use of protective clothing may be important for preventing not only LD but also other tick-borne infections. Nevertheless, these strategies, even used optimally, are likely to prevent only a portion of cases of LD. Other strategies, such as improved vaccines and measures

Table 4. Risk factors for Lyme disease, Connecticut, July 2000 through February 2003

Risk factors	Case-patients, no. (%)	Matched controls, no. (%)	Odds ratio* (95% CI†)	p value
Having an occupational exposure				
Definite	68 (15) N = 462	80 (11) N = 699	1.4 (0.9–2.0)	0.074
Definite and possible	87 (15) N = 566	109 (13) N = 866	1.2 (0.9–1.6)	0.32
Unlikely	38 (28) N = 136	27 (12) N = 222	3.1 (1.6–5.9)	0.0007
Engaging frequently in outdoor activities				
Definite	400 (86) N = 467	598 (83) N = 724	1.2 (0.9–1.7)	0.26
Definite and possible	489 (85) N = 572	745 (83) N = 898	1.2 (0.9–1.6)	0.34
Unlikely	117 (85) N = 137	197 (86) N = 230	0.9 (0.5–1.7)	0.78
Living close to grassy or heavily wooded area				
Definite	444 (95) N = 467	676 (93) N = 724	1.4 (0.8–2.3)	0.23
Definite and possible	546 (95) N = 572	841 (94) N = 898	1.4 (0.9–2.3)	0.18
Unlikely	131 (96) N = 137	218 (95) N = 230	0.9 (0.3–2.6)	0.89
Having pets at home				
Definite	283 (73) N = 386	488 (72) N = 681	1.2 (0.9–1.7)	0.17
Definite and possible	355 (75) N = 472	599 (71) N = 838	1.2 (0.9–1.6)	0.17
Unlikely	81 (77) N = 105	151 (72) N = 210	1.4 (0.8–2.6)	0.27

*All estimates were adjusted for possible confounders (sex, race, receipt of Lyme vaccine, and use of other personal protective measures) with conditional logistic regression.
†CI, confidence interval.

to reduce tick abundance in areas where human exposure to ticks is high, should continue to be pursued.

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Human Bocavirus Infections in Hospitalized Children and Adults

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Studies have reported human bocavirus (HBoV) in children with respiratory tract infections (RTIs), but only occasionally in adults. We searched for HBoV DNA in nasopharyngeal aspirates (NPAs) from adults with exacerbations of chronic bronchitis or pneumonia, from children hospitalized for acute RTIs, and from asymptomatic children during the winter of 2002–2003 in Canada. HBoV was detected in NPAs of 1 (0.8%) of 126 symptomatic adults, 31 (13.8%) of 225 symptomatic children, and 43 (43%) of 100 asymptomatic children undergoing elective surgery. Another virus was detected in 22 (71%) of the 31 HBoV-positive NPAs from symptomatic children. Two clades of HBoV were identified. The pathogenic role of HBoV in RTIs is uncertain because it was frequently detected in symptomatic and asymptomatic children and was commonly found with other viruses in symptomatic children.

Human bocavirus (HBoV) is a newly described human virus closely related to bovine parvovirus and canine minute virus. It is currently classified in the genus *Bocavirus* within the family *Parvoviridae*. This virus was first identified in respiratory tract specimens from Swedish children with lower respiratory tract infections (RTIs) (*1*). Nucleic acid amplification has detected HBoV in respiratory samples of children with acute respiratory disease, with incidence rates ranging from 3% to 19% (*1–23*). However, the pathogenic role of HBoV is uncertain because other viruses have been frequently detected in HBoV-positive children with lower RTIs (range 37%–90%) (*2*,*3*,*7*,*9–11*,*20–22*). The objective of this study was to describe the incidence and clinical manifestations of HBoV infections in children and adults with respiratory tract symptoms, including a control group of children without symptoms.

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Materials and Methods

Study Design

Respiratory samples from adults were obtained from a previous study conducted from December 2002 to April 2003 at 3 university-affiliated hospitals in the province of Quebec, Canada (*24*). Two groups of patients were enrolled: those ≥40 years of age with chronic obstructive pulmonary disease (COPD) who came to emergency departments with exacerbation of their illness (including patients with pneumonia), and those ≥18 years of age without COPD who were admitted to the hospital with a diagnosis of community-acquired pneumonia. Patients were excluded from the study if they came to the hospital >7 days after onset of symptoms.

Respiratory samples from children were obtained from a case-control study, the results of which have been published (*25*). Participants included children ≤3 years of age who were hospitalized from December 2002 to April 2003, at Laval University Hospital Center in Quebec City, Quebec, Canada. Case-patients were children admitted for an acute RTI (mostly bronchiolitis, pneumonitis, and laryngotracheobronchitis) who had a nasopharyngeal aspirate (NPA) collected as part of investigation of their illness. A specific questionnaire was completed at admission by a research nurse in the presence of the parents. At the end of hospitalization, charts of the children were reviewed to collect clinical and laboratory data. Eligible controls were children hospitalized during the same period for any elective surgery (ear, nose, and throat surgeries in 71% of the cases). These children had no concomitant respiratory symptoms or fever at admission. The study nurse obtained a signed consent from parents and an NPA was obtained during surgery. The original studies were reviewed and approved by the ethics committees of all participating healthcare centers.

Laboratory Testing

All pediatric (from case-patients and controls) and adult (case-patients only) NPA specimens were previously analyzed by using a multiplex real-time PCR assay for influenza A and B viruses, human respiratory syncytial virus (hRSV), and human metapneumovirus (hMPV) (*24*,*25*). For symptomatic children, viral cultures and antigen detection assays were performed upon request by the treating physician. Remaining specimens were frozen at –80°C until subsequent HBoV PCR studies.

Nucleic acids were extracted from 200 μL of NPA by using the QIAamp viral RNA Mini Kit (QIAGEN, Inc., Mississauga, Ontario, Canada). A duplex HBoV PCR (TaqMan assay) was used to amplify conserved regions of NP-1 and NS-1 genes as described (*14*), except that the NS-1 forward primer was replaced with primer 5′-TAG TTG TTT GGT GGG ARG A-3′. Probes were labeled with 6-carboxyfluorescein (FAM) or tetrachloro-6-carboxyfluorescein (TET) at the 5′ end and with a quencher at the 3′ end. Amplicons were 81 bp (NP-1) and 74 bp (NS-1), respectively. Duplex amplification was conducted by using 1 μmol/L NS-1 forward primer and 0.4 μmol/L NS-1 reverse primer and the 2 NP-1 primers. Taqman probes were used at concentrations of 0.1 mmol/L for the NP-1 gene and 0.2 mmol/L for the NS-1 gene (*14*). The amplification master mixture consisted of 2.5 mmol/L $MgCl_2$, 3.33 mg/mL bovine serum albumin, 0.2 mmol/L of each of the 4 deoxynucleotide triphosphates (Amersham Biosciences, Uppsala, Sweden), 10 mmol/L Tris-HCl, 50 mmol/L KCl, 0.625 U Promega Taq DNA polymerase (Fisher Scientific, Markham, Ontario, Canada) combined with TaqStart antibody (BD Biosciences Clontech, Palo Alto, CA, USA), and 3 μL DNA in a final volume of 25 μL. PCR amplification (180 s at 94°C and 45 cycles for 10 s at 95°C, 30 s at 58°C, and 30 s at 72°C) was performed in a Smart Cycler thermal cycler (Cepheid, Sunnyvale, CA, USA). A PCR extension step of 5 min at 72°C was performed at the end of the cycling protocol. An HBoV infection was defined by a positive PCR result for NP-1 and NS-1. The duplex assay had a sensitivity of 10 genome copies for NP-1 and NS-1 gene targets on the basis of quantification analysis of positive control plasmids.

Half of the HBoV-positive samples were randomly selected for phylogenetic analysis, which consisted of amplifying and sequencing a 842-bp region of the VP1/VP2 genes as described (*6*). The VP1/VP2 nucleotide sequences from this study, as well as prototype sequence type (ST)1 and ST2 (*1*), were entered into a multiple alignment generated by ClustalW software version 1.83 (www.molecularevolution.org/software/clustalw) and corrected through final visual inspection with the SeqLab application (Wisconsin package version 10.3; Accelrys, San Diego, CA, USA). Phylogenetic analyses were conducted with the MEGA version 3.1 software (*26*) by using the distance method and the neighbor-joining algorithm with Kimura-2 parameters. Topologic accuracy of the tree was evaluated by using 1,000 bootstrap replicates.

Statistical Analysis

Proportions of clinical characteristics in different groups of patients were compared by using the χ^2 test or the Fisher exact test. The Wilcoxon nonparametric test was used to compare age distribution and length of stay. Analyses were performed by using SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Results

HBoV DNA was detected in NPA samples from 1 (0.8%) of 126 symptomatic adults (71 years of age) and from 31 (13.8%) of 225 symptomatic children (mean age 17 months, median age 15 months). However, HBoV was detected more frequently (43%, $p<0.001$) in the 100 asymptomatic control children (mean age 22 months, median age 23 months). Another virus was detected in 22 (71%) of 31 HBoV-positive NPAs from symptomatic children. The virus most commonly co-isolated with HBoV was hRSV (16/31, 52%), followed by influenza A/B (3 cases), hMPV (3 cases), adenovirus (1 case), and parainfluenza virus (1 case). Two children were infected with 2 other viruses in addition to HBoV. The median age of symptomatic children with HBoV infection (15 months) was significantly greater than that of symptomatic children without HBoV infection (8 months; $p<0.0001$). The hospital length of stay was similar for children positive for HBoV DNA (mean 5.1 days, median 4 days) and those negative for HBoV DNA (mean 6.6 days, median 3 days) ($p = 0.9$).

Clinical characteristics of HBoV-positive children are summarized in the Table. There were significantly fewer bronchiolitis episodes in children infected only with HBoV than in children infected only with hRSV ($p<0.0001$). None of the children with single HBoV infections and only 2 (6%) of all 31 HBoV-infected children were admitted to the intensive care unit. In the control group of asymptomatic children who underwent elective surgery, ear, nose, and throat surgery was more frequently performed in children with HBoV infections (36/43, 84%) than in children without HBoV infections (35/57, 61%) ($p = 0.014$). Ear, nose, and throat elective surgeries consisted mainly of myringotomies, adenoidectomies, and tonsillectomies.

The 1 adult with an HBoV infection was a 71-year-old man (a smoker) who came to the hospital for a COPD exacerbation and was treated with systemic corticosteroids and antimicrobial drugs. No other microbiologic agents (bacteria or viruses) could be identified in his sputum or NPA. He was hospitalized for 11 days.

Sequence analysis of the HBoV VP1/VP2 genes performed on ≈50% of HBoV-positive specimens showed 2

Table. Clinical characteristics of symptomatic children with respiratory tract infections*

Characteristic	HBoV, all infections (n = 31)	HBoV, single infections (n = 9)	hRSV, single infections (n = 97)	hMPV, single infections (n = 12)	Influenza A/B virus, single infections (n = 3)
Pneumonia, no. (%)	13 (42)	4 (44)	17 (18)	1 (8)	1 (33)
Bronchiolitis, no. (%)†	13 (42)	1 (11)	83 (86)	5 (42)	2 (67)
Laryngotracheobronchitis, no. (%)	0	0	0	0	0
Otitis media, no. (%)	19 (61)	4 (44)	58 (60)	7 (58)	1 (33)
Median (mean) length of stay, d	3 (6.6)	3 (3.6)	4 (4.6)	3 (6.7)	4 (4.0)
Admission to ICU, no. (%)	2 (6)	0	10 (10)	0	0
Underlying cardiopulmonary disorders, no. (%)‡	3 (10)	0	11 (11)	3 (25)	3 (100)
Prematurity, no. (%)	2 (6)	0	10 (10)	0	0

*HBoV, human bocavirus; hRSV, human respiratory syncytial virus; hMPV, human metapneumovirus; ICU, intensive care unit.
†p<0.0001 for comparison of single infections.
‡p = 0.0014 for comparison of single infections.

distinct clades of viruses (Figure). These genotypes clustered with the original strains described by Allander et al. (ST1, GenBank accession no. DQ000495, and ST2, GenBank accession no. DQ000496) (*1*). There was no temporal link between the clades because both were equally distributed throughout the study period. No obvious relationship was found between clades and the presence or absence of symptoms.

Discussion

Results from our study indicate that HBoV was rarely detected in adults with respiratory symptoms but was frequently detected in symptomatic and asymptomatic children during the 2002–2003 winter season. HBoV was detected in NPA samples from 1 (0.8%) of 126 symptomatic adults, 31 (13.8%) of 225 symptomatic children, and 43 (43%) of 100 asymptomatic children. Another virus was detected in 22 (71%) of 31 HBoV-positive samples from symptomatic children. Overall, these data do not support a pathogenic role for HBoV in acute RTIs in children.

The full spectrum of clinical diseases associated with HBoV infections and the epidemiology of this new parvovirus are not fully understood. This is particularly true for adult patients in whom few studies have been performed. Allander et al. (*1*) found no HBoV DNA in 112 culture-negative NPA samples from adults with respiratory symptoms. Bastien et al. (*5*) reported an overall rate of infection of 1.5% in respiratory samples negative for other viruses, with no differences between age groups. Maggi et al. (*16*) reported only 1 HBoV-positive sample from an adult with lymphoma in 62 bronchoalveolar lavages (BALs). These investigators also tested 22 nasal swabs from adults with persistent asthma symptoms and found no samples positive for HBoV. Fry et al. (*10*) identified HBoV DNA in 1% of adults >20 years of age hospitalized with pneumonia in Thailand. Kupfer et al. (*27*) described a case of HBoV infection associated with severe atypical pneumonia in a patient with non-Hodgkin lymphoma who was also infected with cytomegalovirus in a BAL sample. We found 1 case of HBoV infection in an adult, which represented 0.8% of the tested population. The HBoV-positive adult did not show immunosuppression but was treated with corticosteroids for a COPD exacerbation. Overall, our results are consistent with those of previously described studies and support the fact that HBoV infection is rare in adults but may occur more frequently in those with other illnesses or immunosuppression.

Studies have reported HBoV DNA in 3%–19% of children with RTIs. Rates of detection tend to be higher in children <1 year of age (*4*,*10*). The incidence of HBoV infections also tends to be higher in samples from the lower respiratory tract, such as NPA or BAL (4.4%–19%) (*2*,*7*,*9*,*11*,*13*,*19*,*21*,*22*), than in nasal swabs (1%–6%) (*10*,*15*,*16*,*18*). The percentage of co-pathogens in our HBoV-positive children (71%) was comparable with those reported in the literature, with rates of co-infections ranging from 35% to 90% (*2*,*3*,*7*,*9–11*,*20–22*). Moreover, co-infecting viruses detected in conjunction with HBoV in our population were similar to those described in other studies, i.e., hRSV, influenza A virus, and adenovirus (*11*,*23*).

The high frequency of HBoV detection (43%) in our asymptomatic children contrasts with the results of the few other studies that included a control group of asymptomatic children. Fry et al. (*10*) detected HBoV DNA in only 1% of nasal swabs from asymptomatic patients. Maggi et al. (*16*) did not detect HBoV DNA in nasal swabs from 51 asymptomatic children (including 30 healthy infants with a mean age of 6 months and 21 preadolescent healthy children with a mean age of 12.8 years). However, these studies analyzed nasal swabs instead of NPA or BAL samples for HBoV detection, which may result in lower rates of viral detection, as shown in symptomatic persons. Allander et al. (*2*) did not detect HBoV in any of 64 asymptomatic children (median age 4.1 years, range 5 months to 14 years) but used nasal swabs in asymptomatic patients and NPA samples in symptomatic patients. Furthermore, their control group was also older than our population (mean 18.6 months, median 18 months). Kesebir et al. (*12*) did not detect any HBoV

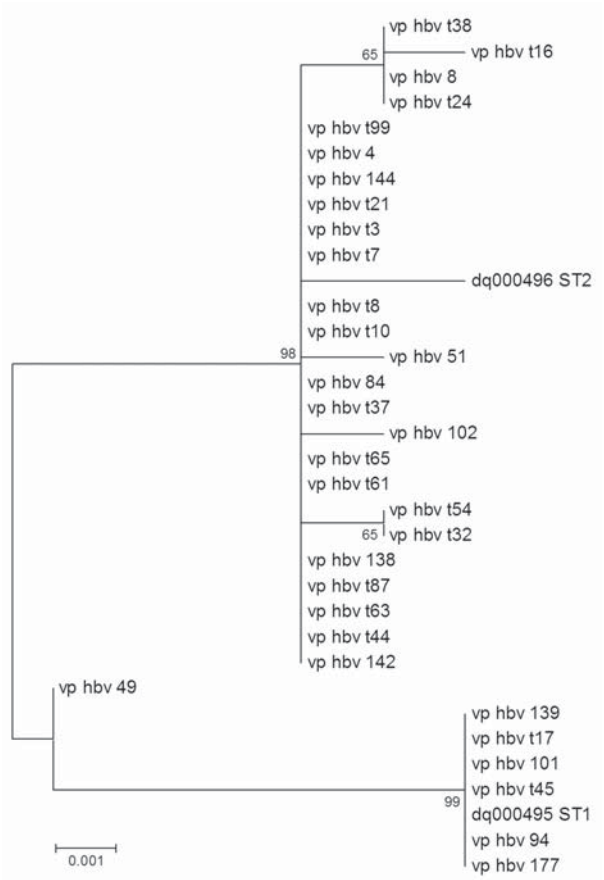

Figure. Phylogenetic tree of human pediatric bocavirus strains from Quebec City, Quebec, Canada. Patient numbers beginning with the letter t indicate asymptomatic (control) children. Strains from Sweden (sequence type [ST] 1, GenBank accession no. DQ000495, and ST2, GenBank accession no. DQ000496) are included (*1*). Numbers along branches are bootstrap values from 1,000 replicates. Scale bar shows 1 substitution for every 1,000 nucleic acid residues.

DNA in nasal washes from 96 asymptomatic children <2 years of age seen at a clinic compared with 22 (5.2%) of 425 various samples from symptomatic children sent to a hospital clinical laboratory. None of the previous studies used a control group consisting of children matched for age and week of admission and analyzed the same type of respiratory samples for cases and controls.

Our positive results for HBoV were confirmed by using 2 sets of PCR primers targeting different genes (NP1 and NS1) in a duplex PCR assay and by subsequent testing with a third set of primers (VP1/VP2) for sequencing. Also, sample preparation and PCR amplification were performed in separate laboratory areas following the stringent quality control program of our institution. Thus, it is unlikely that our positive results were due to PCR cross-contamination. Our method was also very sensitive (detection limit = 10 genome copies), which probably enabled an increased infection rate compared with previous reports. We cannot exclude the possibility that prior RTIs (in the few weeks preceding sampling) occurred in our asymptomatic children hospitalized for an elective surgery or that HBoV could establish a prolonged infection in children compared with other respiratory viruses. However, the 3× higher detection rate in controls than in symptomatic children make these explanations unlikely. We did not quantify HBoV DNA load in samples from our study, which could have been different between asymptomatic and symptomatic children. Nevertheless, we detected hRSV, hMPV, and influenza virus RNA in <1% of the same NPA samples from those asymptomatic children compared with a rate of 43% for HBoV DNA (*25*). At the very least, our results should raise concerns about the pathogenic role of HBoV in children.

We detected 2 HBoV genotypes circulating at the same time in both symptomatic and asymptomatic children during the winter of 2002–2003 in Quebec. This result is consistent with findings of other groups from North America and Europe during 2002–2004 and highlights the fact that HBoV lineages do not appear to be geographically clustered (*1*,*6*,*9*,*12*). The seasonality of HBoV infection is still a matter of debate, but it seems to involve primarily the colder months of the year (*9*,*20*,*21*). However, most studies, including ours, were performed during the typical respiratory virus season, which may have introduced a bias. Additional studies are needed to address the prevalence of HBoV outside the respiratory virus season and its role in nonrespiratory syndromes. Moreover, the possibility that this virus might be transmitted and isolated in the respiratory tract, but could cause viremia and other clinical syndromes such as gastroenteritis, should be investigated. Vicente et al. analyzed 527 stool samples from children with gastroenteritis and no respiratory symptoms and found a positivity rate of 9.1% for HBoV (with a co-infection rate of 58%) (*22*).

In conclusion, our study shows that HBoV was frequently detected in both symptomatic and asymptomatic children during the winter of 2002–2003 in Quebec City. Conversely, this virus was rarely found in the adult population during the same period. Further studies are needed to establish whether this recently described parvovirus is pathogenic by using well-matched control groups and sequential samples to detect viral persistence.

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Dr Longtin is a resident in infectious diseases and medical microbiology at Laval University in Quebec City, Quebec, Canada. His research interests include the epidemiology of emerging viruses and pharmacokinetic studies of antiretroviral agents.

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Genetic Determinants of Virulence in Pathogenic Lineage 2 West Nile Virus Strains

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We determined complete genome sequences of lineage 2 West Nile virus (WNV) strains isolated from patients in South Africa who had mild or severe WNV infections. These strains had previously been shown to produce either highly or less neuroinvasive infection and induced genes similar to corresponding highly or less neuroinvasive lineage 1 strains in mice. Phylogenetic and amino acid comparison of highly and less neuroinvasive lineage 2 strains demonstrated that the nonstructural genes, especially the nonstructural protein 5 gene, were most variable. All South African lineage 2 strains possessed the envelope-protein glycosylation site previously postulated to be associated with virulence. Major deletions existed in the 3′ noncoding region of 2 lineage 2 strains previously shown to be either less or not neuroinvasive relative to the highly neuroinvasive strains sequenced in this study.

West Nile virus (WNV) is endemic to Africa, Asia, Europe, and Australia and was introduced into the Western Hemisphere in 1999. In the Northern Hemisphere, an apparent increase in human case fatality rates, neurologic infections, and horse and bird deaths due to WNV has raised the question whether WNV strains with increased pathogenicity have emerged in the Northern Hemisphere, or whether the virulence of the virus and the severity of the disease are underestimated in South Africa.

Two major phylogenetic lineages of WNV have been demonstrated: lineage 1 includes viruses from North Africa, Europe, Asia, the Americas, and Australia (Kunjin virus); lineage 2 consists exclusively of viruses from southern Africa and Madagascar. The increase in illness and death from WNV lineage 1 strains relative to lineage 2 strains led to the supposition that lineage 1 strains are highly pathogenic while lineage 2 strains endemic to Africa are of low virulence (*1,2*). However, it was subsequently demonstrated in South Africa that lineage 2 strains may also cause severe disease (*3*). Furthermore, experiments using mice demonstrated marked differences in neuroinvasive phenotype that did not correlate with lineage, which suggests that highly and less neuroinvasive phenotypes exist in both lineages (*2,4*). Host gene expression studies indicated that similar genes are induced by highly neuroinvasive lineage 1 and 2 strains (*4*). Therefore, the perceived virulence of WNV in recent epidemics probably reflects high medical alertness, active surveillance programs, and the emergence and reemergence of existing strains of WNV in locations with immunologically naive populations (*3*). Recently, a lineage 2 strain was isolated from a goshawk fledgling that died of encephalitis in Hungary, which suggests that lineage 2 strains may also be spread by migratory birds outside of Africa (*5*).

Lineage 1 viruses that have phenotypes of reduced virulence in mice and inefficient growth in culture have been identified in Mexico. Mutations leading to loss of envelope (E) protein glycosylation together with mutations in the nonstructural (NS) protein genes may be associated with attenuation of these viruses (*6*). Comparisons between the prototype Uganda strain (B956) and a variant of this strain, which was obtained by molecular mutation (B956D117B3), showed changes in the E and NS genes, which resulted in reduced virulence in mice (*7*). These attenuations could not, however, be correlated with clinical disease in humans because these strain were either isolated from birds or modified in culture.

The NS4B protein may play an important role in virulence phenotype determination (*6,8–10*), predicted to be involved in viral replication and evasion of host innate

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immune defenses (*8*). Substitution of cysteine at position 102 with serine (Cys102Ser) led to the formation of a temperature-sensitive phenotype at 41°C as well as attenuation of the neuroinvasive and neurovirulent phenotypes in mice (*8*). An adaptive mutation (E249G) in the NS4B gene resulted in reduced RNA synthesis in host cells (*9*). An in vitro study compared infectious clones of the NY99 strain, which is highly virulent in American crows, with a Kenya strain (KEN-3829), which is less virulent for American crows. After 72 days at 44°C, reduction in viral RNA production by the KEN-3829 strain was 6,500-fold, compared with the NY99 strain reduction of 17-fold. This finding suggested that efficient replication at high temperatures, as occurs in American crows, could be an important virulence factor that determines the pathogenic phenotype of the NY99 strain (*10*).

To further investigate the molecular determinants of virulence of lineage 2 WNV strains, we sequenced the genomes of highly and less neuroinvasive lineage 2 strains that were isolated from patients in South Africa and that had previously been characterized with respect to gene expression and pathogenicity (*4*). These complete genome sequences of highly neuroinvasive lineage 2 WNV strains enable comprehensive comparison with highly and less neuroinvasive lineage 1 strains.

Materials and Methods

Virus Strains

South African WNV isolates SPU116/89, SA93/01, SA381/00, and H442 were obtained from the Special Pathogens Unit, National Institute for Communicable Diseases, South Africa, as freeze-dried mouse brain passages 2–4. They were replicated by 1 passage in Vero cells for this study.

RNA Amplification

Viral RNA was extracted from cell culture supernatant with the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis, 10 μL of RNA and 0.4 μg of random hexanucleotides (Roche Diagnostics, Mannheim, Germany) were incubated at 65°C for 10 min before cooling on ice. Then 1× Expand Reverse Transcriptase buffer, 100 mmol/L dithiothreitol, 200 μmol of each deoxynucleotide triphosphate, 20 U RNase Inhibitor and 50 U Expand Reverse Transcriptase (Roche Diagnostics) were added and incubated at 30°C for 10 min, followed by 1 h at 43°C. For PCR amplification, 10 μL of the cDNA reaction was added to the PCR master mix consisting of 3.75 U of Expand High Fidelity Polymerase and 30 pmol of each specific primer (primer sequences available on request) and cycled as follows: 94°C for 2 min (94°C for 15 s, followed by primer-specific annealing temperature for 30 s, 72°C for 2 min) × 35 and 72°C for 7 min. Expand Long Template PCR Polymerase (Roche Diagnostics) was used for products >2 kb with 300 μmol of each dNTP, 1× buffer, and 30 pmol of each specific primer and cycled at 94°C for 2 min (94°C for 10 s, 50°C for 30 s, 68°C for 3 min) × 10; followed by 30 cycles of 94°C for 15 s, 50°C for 30 s, 68°C for 5 min plus 5 s per cycle, and 72°C for 7 min.

DNA Sequencing

PCR products were purified with Wizard SV gel and PCR clean-up system (Promega, Southampton, UK). DNA cycle sequencing was performed with the BigDye Terminator V3.1 kit and analyzed on an ABI PRISM 3100/3130 genetic analyzer (both from Applied Biosystems, Foster City, CA, USA).

Sequence Analysis

Genome editing and assembling were performed by using Vector NTI 9.1.0 (Invitrogen, Carlsbad, CA, USA); multiple sequence alignments, with ClustalW (*11*); and amino acid analysis, with GeneDoc for Windows (*12*). Amino acid changes considered to have a potential effect on the secondary structure of the proteins included substitution of hydrophilic for hydrophobic amino acids or vice versa and substitutions of cysteine, glycine, and proline residues (*12*).

Comparisons are relative to the top sequence (SA381/00); numbering refers to the sequence position of isolate SA381/00. Neighbor-joining trees were drawn with MEGA version 3.1 (*13*) by using the Kimura-2 distance-parameter and a bootstrap confidence level of a 1,000 replicates. Nucleotide and amino acid p-distances (the number of pairwise nucleotide or amino acid differences divided by the total number of nucleotides or amino acids in the sequenced region) were calculated by using MEGA version 3.1. Signalase cleavage predicted scores were calculated with AnalyzeSignalase 2.03 (*14*).

Results

Strain Characteristics

Four lineage 2 WNV strains isolated from patients in South Africa who had mild or severe WNV infections were selected for genome sequencing. Phenotypic pathogenicity data for these strains (H442, SPU116/89, SA93/01, SA381/00) in humans and mice are summarized in Table 1. Detailed clinical data for all 4 strains have been described by Burt et al. (*3*), and mouse neuroinvasive experiments and gene expression data for H442, SPU 116/89, and SA381/00 have been described by Venter et al. (*4*). Strain SA93/01 has been shown to be highly neuroinvasive in a mouse model (M. Venter, unpub. data), similar to SPU116/89 and H442 strains, whereas SA381/00 has been classified as being of low neuroinvasive phenotype in mice. H442

Table 1. Characteristics and origin of West Nile virus strains included in this investigation*

Strain, year of isolation	Passage	Source	Location	Syndrome	Outcome	L	Neuro	Glyco	Ref	GenBank
SPU116/89, 1989	Mouse 3	Human	SA	Necrotic hepatitis	Died	2	High	NYS†	This study	EF429197
SA93/01, 2001	Mouse 1	Human	SA	Fever, rash, myalgia, encephalitis	Survived	2	High	NYS†	This study	EF429198
SA381/00, 2000	Mouse 1	Human	SA	Fever, rash, myalgia, arthralgia	Survived	2	Mild	NYS†	This study	EF429199
H442, 1958	Mouse 2	Human	SA	Fever, rash, myalgia, arthralgia	Survived	2	High	NYS†	This study	EF429200
B956, 1937	Mouse 2	Human	Uga	Febrile disease	Survived	2	Mild	Deletion of entire motif	(*7*)	AY532665
B956D117B3,‡ 1937	Unknown	Human	Uga	Febrile disease	Survived	2	Less than B956	Deletion of entire motif	(*15*)	M12294
Madagascar-AnMg798, 1978	Unknown	Parrot§	Mad	NA	Died	2	None	NYP	(*16*)	DQ176636
NY 385–99, 1999	Vero 2	Human	USA	Unknown	Unknown	1	High	NYS†	(*17*)	DQ211652
NY-385–99 Clone TYP-9376, 2005	Hamster	Hamster	USA	NA	NA	1	None¶	NYS†	(*18*)	AY848697
NY-385–99 Clone 9317B, 2005	Hamster	Hamster	USA	NA	NA	1	None¶	NYS†	(*18*)	DO66423
TM171–03, 2003	Vero 1	Common raven	Mex	Unknown	Died	1	None¶	NYP	(*19*)	AY660002
MRM61C, 1960	NA	Mosquito#	Aus	NA	NA	1	None	NYF	(*20*)	D00246

*L, lineage; Neuro, neuroinvasiveness in mice; Glyco, glycosylation of envelope protein; Ref, reference no.; GenBank, GenBank accession no.; SA, South Africa; Uga, Uganda; Mad, Madagascar; NA, not applicable; USA, United States; Mex, Mexico; Aus, Australia.
†Strains that are glycosylated in the envelope protein positions 154–156.
‡A descendant of B956.
§*Coracopsis vasa*.
¶Attenuated laboratory strains.
#*Culex annulirostris*.

and SA381/00 caused fever, rash, myalgia, and arthralgia in human patients; SA93/01 caused nonfatal encephalitis in 2, and SA116/89 caused fatal hepatitis (*3*).

The 4 South African strains were compared with strains that were known to be highly or less neuroinvasive in mice or that had been reported to be highly pathogenic or attenuated. Lineage 2 strains for which both full genome sequences and neuronvirulence data in mice were available included isolate B956D117B3 (*21*) and Madagascar strain AnMg798. B956D117B3 is a passaged clone of reduced virulence (*7*) of the prototype strain (B956), which was originally associated with fever in a patient and was neurotropic in mice (*22*); AnMg798 is not neuroinvasive (*2*). Lineage 1 strains included the highly pathogenic and neuroinvasive NY385–99 strain (*2*), the attenuated non-neuroinvasive strain TM171–03 isolated in Mexico in 2003 (*19*), hamster-passaged attenuated clones of NY-385–99 (clone TYP-9376 and clone 9317B) (*18*), and a non-neuroinvasive Kunjin virus strain MRM61C (Table 1).

Phylogenetic Analysis

Phylogenetic analysis confirmed that the South African strains described here belong within lineage 2 (Figure 1). SA93/01 and SPU116/89 clustered together; H442 and SA381/00 were on separate branches within lineage 2 with respect to the full genome sequences or with respect to individual E, NS3, and NS5 genes (data not shown). Although the Indian strain clustered with lineage 1, p-distance analysis suggested that it was as distant to the lineage 1 strains (20% differences) as to the lineage 2 strains (21%–22%) relative to <5% differences within lineage 1C and 12% differences between 1A and 1B. It was therefore termed lineage 5, as suggested by Bondre et al. (*23*).

Genome Sequences and Distance Analysis

The complete genome sequences of strains H442, SPU116/89, SA381/00, and SA93/01 were deposited in GenBank (accession nos. EF429197–200). The termini were amplified with primers designed from other lineage 2 full genome sequences. If one assumes that the 5′ and 3′

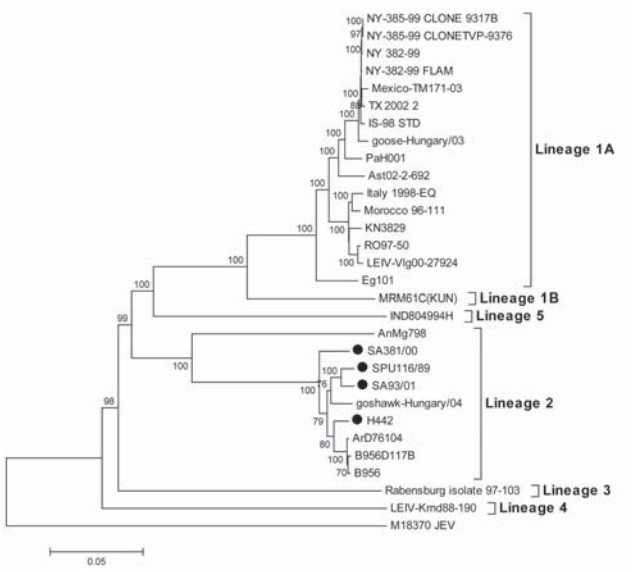

Figure 1. Phylogenetic analysis of full genome nucleotide sequences of lineage 1 and 2 strains of West Nile virus. The tree was constructed by using MEGA version 3.1 (*13*) with the neighbor-joining method and Kimura 2-parameter distance matrix. A bootstrap confidence level of 1,000 replicates was used. South African strains sequenced in this study are indicated by a black circle. GenBank accession nos. are as follows: NY-385–99 clone 9317B (DO66423), NY-385–99 clone TVP-9376 (AY848697), NY 385–99 (DQ211652), NY-382–99 FLAM (AF196835), IS-98 STD (AF481864), Mexico-TM171–03 (AY660002), TX 2002 2, (DQ 164205), goose-Hungary/03 (DQ 118127), Eg 101 (AF 260968), RO97–50 (AF260969), Morocco 96–111 (AY701412), Italy 1998-Eq (AF 404757), KN3829 (AY262283), LEIV-Vlg00–27924 (AY278442), PaH001 (AY268133), Ast02–2-696 (DQ411035), MRM61C (KUN) (D00246), IND804994H (DQ 256376), AnMg798 (DQ 176636), SA381/00 (EF429199), H442 (EF429200), goshawk-Hungary/04 (DQ 116961), SPU116/89 (EF429197), SA93/01 (EF429198), B956D117B3 (M12294), B956 (AY532665), ArD76104 (DQ 318019), Rabensburg isolate 97–103 (AY765264), LEIV-Krnd88–190 (AY277251), and M18370 JEV (M18370).

termini are identical in length to other published strains, these genomes were 11052 nt (SPU116/89, SA381/00, and SA93/01) and 11051 nt (H442) long. South African strains had overall nucleotide p-distances of 0.0278 (97.2% similarity) to each other (Table 2), with <1% amino acid differences over the complete genome despite having been isolated as many as 50 years apart. The highest percentage of amino acid differences in the individual proteins of the lineage 2 strains were in the NS proteins, especially the NS5 protein. Table 3 shows the differences between individual proteins of the South African lineage 2 strains.

The 2 strains from North America, New York (NY-385–99) and Mexico (TM171–03), were similarly conserved. In contrast, the Madagascar strain, AnMg798, differed by >3% at the amino acid level from all lineage 2 strains from South Africa or Uganda B956D117B3, and the lineage 1 and 2 strains differed by >6% amino acids from each other.

Amino Acid Differences between Highly and Less Neuroinvasive Strains

Few amino acid differences were observed between the structural proteins of the South African strains (Figure 2). SA381/00 had only 1 difference in the premembrane (prM) protein at position 105 relative to the highly neuroinvasive strains (Ala105Val) (Figure 2). Two differences, (Ala54Gly and Thr70Pro) could result in structural changes in the E protein of H442, which was isolated 50 years earlier than strains SPU116/89, SA93/01, and SA381/00. The attenuated lineage 2 strain B956D117B3 and the nonneuroinvasive Madagascar strain AnMg798 contained differences in the glycosylation site of the E protein relative to the South African strains (residues 154–157 deleted in B956D117B3, and Ser156Pro in AnMg798). Either of these changes would prevent glycosylation. Further substitutions of hydrophilic amino acids for proline and glycine residues with potentially structural implications were found in AnMg798 at positions 156, 199, and 230.

The NS3, NS4A/B, and NS5 proteins were the most variable viral proteins. In strain SA381/00, the least virulent of the 4 strains, a hydrophobic amino acid in contrast to a hydrophilic amino acid (Ser160Ala) and Arg298Gly could alter the structure of the SA381/00 NS3 protein. In the highly pathogenic strain SPU116/89, a hydrophobic-to-hydrophilic mutation (Ala79Thr) in NS4B is found relative to the other strains. Other amino acid changes with potential structural implications were for strain B956D117B3 at positions 18 and 145 of the NS4A gene and 14 of the NS4B gene and for strain AnMg798 at positions 14 and 27 in the NS4B gene of (Figure 2).

The NS5 protein was the most variable. Several positions were identified where the South African strains associated with mild infections (SA381/00 and H442) and the 2 other lineage 2 strains (AnMg798,B956D117B3) associated with reduced virulence in mice had the same amino acid changes relative to strains that caused severe disease (SPU116/89 and SPU93/01). These included hydrophilic versus hydrophobic amino acids in position 614 and hydrophobic (mild) versus hydrophilic (pathogenic) in positions 625 and 626 of the NS5 protein. SPU116/89, isolated from a patient with necrotic hepatitis, was found to have amino acid changes that affect the hydrophobicity of the NS5 protein relative to all other strains in positions 197, 623, 635, 641, and 643.

Noncoding Regions

Approximately 98.6% identity existed between the 5′ noncoding regions of SA381/00 and the 3 remaining South African strains; the other strains were 100% conserved. For

Table 2. Percentage of amino acid and nucleotide differences when comparing the entire genome of selected West Nile virus strains*

Strain	SA381/00	H442	SPU116/89	SA93/01	B956D117B3	B956	ANMg798	NY-385–99	NY-385–99 Clone TYP-9376	NY-385–99 Clone 9317B	TM171–03	MRM61C
SA381/00		2.4	3.6	3.7	3.1	2.9	15.8	20.6	20.6	20.6	20.6	20.5
H442	0.7		2.7	3.0	1.8	1.6	15.6	20.5	20.6	20.6	20.5	20.6
SPU116/89	0.9	0.7		1.3	2.0	1.9	16.0	20.8	20.9	20.9	20.8	20.7
SA93/01	0.8	0.7	0.6		2.3	2.2	15.7	20.8	20.9	20.9	20.8	20.6
B956D117B3	1.0	0.8	1.0	0.9		0.3	15.7	20.7	20.7	20.7	20.7	20.6
B956	0.7	0.6	0.7	0.7	0.7		15.8	20.7	20.7	20.7	20.7	20.6
AnMg798	3.4	3.3	3.5	3.4	3.5	3.4		21.5	21.5	21.5	21.4	21.2
NY-385–99	6.0	5.9	6.1	6.1	6.1	6	6.6		0.1	0.1	0.4	11.7
NY-385–99 Clone TYP-9376	6.1	6.0	6.2	6.2	6.2	6.1	6.7	0.1		0	0.5	11.8
NY-385–99 Clone 9317B	6.1	6.0	6.2	6.2	6.2	6.1	6.7	0.1	0.0		0.5	11.8
TM171–03	6.0	5.9	6.1	6.1	6.1	6	6.7	0.1	0.2	0.2		11.8
MRM61C	6.5	6.5	6.7	6.6	6.6	6.5	7.1	2.4	2.4	2.4	2.4	

*The lower left matrix corresponds to amino acid sequences, and the upper right matrix corresponds to nucleotide sequences.

the 3′ noncoding regions, the overall identity was 98.5% (99% between SPU381/00 and H442 and 98% between SPU116/89 and SA381/00). Noteworthy nucleotide differences in the 3′ noncoding regions were a 2-nt deletion at nt 10439 and nt 10440 in strain H442 and a 76-bp deletion in the 3′ noncoding region from nt 10404 through nt 10479 in the attenuated strain B956D117B3, which was not present in the prototype strain (B956) or in any of the South African strains. Strain AnMg798 had deletions overlapping those of strain B956D117B3 at position 10411 to 10487 and from 10501 to 10512 and 10951 (Figure 2, panel B). The sequence of the AnMg798 strain is incomplete in GenBank and ended at position 10866 (*16*).

Envelope-Protein Glycosylation Motif

The E protein glycosylation motif previously identified in lineage 1 at positions 154–156 (NYS) (*6*) was present in all 4 South African strains. However, as a result of a proline substitution at position 156, the site was not predicted to be glycosylated in strain AnMg798. The glycosylation motif is deleted completely in strains B956 and B956D117B3 (Table 1).

Cleavage Sites

Signalase prediction algorithms were used to analyze the signal peptidase cleavage sites (*14*); no differences were found in cleavage efficiency between the highly and less pathogenic strains (Table 4). The only meaningful difference was observed in the capsid (C)–PrM cleavage region, as indicated by the Student *t* test probability calculated in Table 4, where the lineage 2 strains were predicted to be cleaved more efficiently than lineage 1 stains. Only slight differences were apparent in the PrM-E site; no differences were apparent in any other cleavage regions between lineage 1 and 2 strains.

Discussion

Phylogenetic and p-distance analyses suggested that relationships between WNV strains were influenced by geographic rather than temporal factors (Figure 1, Tables 2, 3). Four South African strains isolated over 50 years differed from each other by an average of only 3% of nucleotides but from the AnMg798 (Madagascar) strain by 21%.

The WNV genome consists of a 5′ noncoding region, a single open reading frame coding for 3 viral structural proteins (C, M, and E) and 7 NS proteins, and a 3′ noncoding region. The E and membrane (M) proteins are associated with host range, tissue tropism, replication, assembly, and the stimulation of the B- and T-cell immune responses; replication functions are associated with the NS proteins, which may also modulate responses to viral infection (*6*). The E protein is the viral hemagglutinin that mediates virus–host cell binding and elicits most of the virus neutralizing antibodies and serotype specificity of the virus (*1,24,25*).

In this study, differences between highly and less neuroinvasive lineage 2 stains were identified in the noncoding regions, which may potentially affect enzyme binding sites and replication efficiency (Figure 2, panel B). It has been postulated that the 3′ stem loop structure may function as a translation suppressor (*26*) and that nucleotide sequence variation in the 3′ noncoding region of different dengue strains may have evolved as a function of transmission or replication ability in different mosquito and nonhuman primate/human host cycles (*27*). A 76-bp deletion in the 3′ noncoding region is present in strains B956D117B3 and AnMg798 relative to the South African strains. This deletion is not present in the original neurotropic mouse brain isolate of the B956 Uganda strain, which has recently been resequenced (*7*). Strain B956D117B3, a descendent of the original B952 isolate, has been shown to be less virulent

Table 3. Percentage amino acid and nucleotide differences when comparing individual proteins of selected West Nile virus strains*

Strain	SA381/00	H442	SPU116/89	SA93/01
		Capsid		
SA381/00				
H442	0			
SPU116/89	0	0		
SA93/01	0	0	0	
		Envelope		
SA381/00				
H442	0.8			
SPU116/89	0.2	0.6		
SA93/01	0.2	0.6	0	
		NS2A/B		
SA381/00				
H442	0.6			
SPU116/89	0.3	0.8		
SA93/01	0.3	0.8	0	
		NS4A/B		
SA381/00				
H442	0.2			
SPU116/89	0.2	0.5		
SA93/01	0	0.2	0.2	
		prM		
SA381/00				
H442	0.6			
SPU116/89	0.6	0		
SA93/01	0.6	0	0	
		NS1		
SA381/00				
H442	0.9			
SPU116/89	0.9	0		
SA93/01	1.1	0.3	0.3	
		NS3		
SA381/00				
H442	0.8			
SPU116/89	0.6	0.5		
SA93/01	0.6	0.5	0	
		NS5		
SA381/00				
H442	0.8			
SPU116/89	2.1	1.5		
SA93/01	1.9	1.3	2.0	

*prM, premembrane; NS, nonstructural.

than the original B956 strain. The absence of this deletion in all of the neuroinvasive lineage 2 strains isolated from clinical cases warrants further investigation of the role of the region in the pathogenicicty of WNV.

The genetic stability observed in the surface E and M proteins of lineage 2 strains suggests an absence of immune-driven selection. Only the H442 strain, isolated 50 years before the other strains, had 2 substitutions in the E gene with potential structural implications (Figure 2). The absence of a putative E protein glycosylation site at positions 154–156 of the E protein (NYS) has previously been associated with reduced virulence in mice (*19*). This glycosylation motif was present in all the South African strains, including the less neuroinvasive strain SA381/00. However, the prototype lineage 2 strain B956D117B3 and the non-neuroinvasive lineage 1 and 2 strains MRM61C and AnMg798 were not glycosylated. This finding further emphasizes that glycosylation of the E protein is not the only determining factor for virulence.

Most substitutions were found in the NS proteins, in particular NS3, NS4A/B, and NS5. The NS3 protein is part of the protease complex, which is important for cleavage of the polyprotein and may affect virulence; it has been suggested that less efficient cleavage results in delayed virus assembly and release, enabling the host immune system to clear infection (*28*). The NS3 protein of the less neuroinvasive strain, SA381/00, manifested hydrophobic and hydrophilic changes, which could lead to structural changes that affect function and, by implication, virulence. The highly neuroinvasive strain SPU116/89 had mutations that may al-

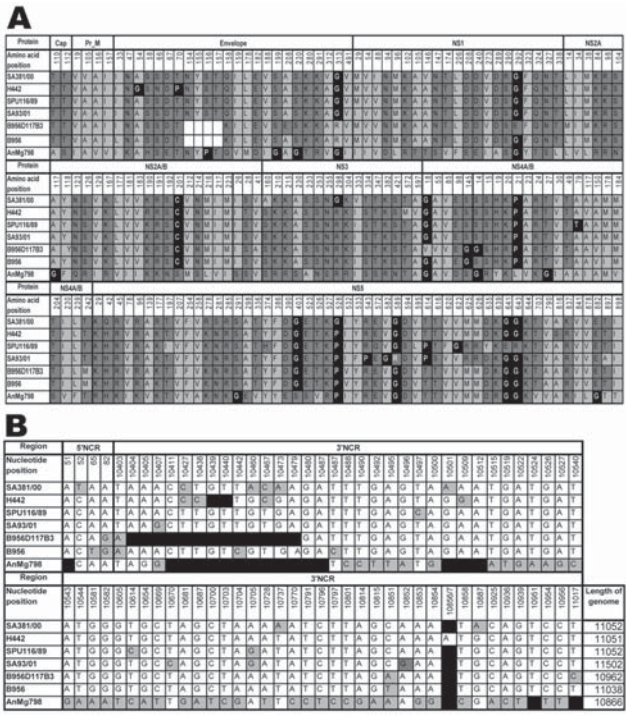

Figure 2. A) Amino acid differences between South African lineage 2 strains of West Nile virus (WNV) strains sequenced in the present study and previously published lineage 2 strains. Strain SA381/00 is less neuroinvasive than the highly neuroinvasive strains SA93/01, H442, and SPU116/89. Light gray, hydrophobic amino acids; dark gray, hydrophilic amino acids; black, structural-determining amino acids; white blocks, deletion of the glycosylation site in the envelope protein of the B956D117B3 strain. Numbering is according to the SA381/00 genome position for specific genes. Cap, capsid; prM, premembrane; NS, nonstructural. B) Nucleotide differences in the noncoding 5′ and 3′ regions of lineage 2 strains. Numbering is according to WNV strain SA381/00. Black, deletions; gray, nucleotide differences. The length of each genome is given in the last column. (Strain AnMg798 is incomplete in the GenBank database and may thus be longer than indicated.)

Table 4. Summary of cleavage scores predicted for cleavage junctions of proteins of West Nile virus strains*

	SA381/00	H442	SPU116/89	SA93/01	B956D1 17B3	AnMg798	NY-385–99	NY-385–99 clone TYP-9376	NY-385–99 clone 9317B	TM171–03	MRM61C	p value†
Between capsid and premembrane proteins												
G	+3.69	+3.69	+3.69	+3.69	+3.69	+4.00	–0.49	–0.49	–0.49	–0.49	–1.85	0.00005
A‡	+9.37	+9.37	+9.37	+9.37	+9.37	+8.01	+5.93	+5.93	+5.93	+5.93	+7.37	0.00004
V	–9.14	–9.14	–9.14	–9.14	–9.14	–7.8	–9.52	–9.52	–9.52	–9.52	–10.32	0.02235
Between premembrane and envelope proteins												
Y	–10.15	–10.15	–10.15	–10.15	–10.15	–9.12	–9.12	–9.12	–9.12	–9.12	–9.45	0.00433
S‡	+11.27	+11.27	+11.27	+11.27	+11.27	+12.42	+12.42	+12.42	+12.42	+12.42	+11.50	0.01728
F	–5.37	–5.37	–5.37	–5.37	–5.37	–4.78	–4.78	–4.78	–4.78	–4.78	–5.27	0.01977
Between envelope protein and nonstructural protein 1												
H	–9.01	–9.01	–9.01	–9.01	–9.01	–9.71	–9.01	–9.01	–9.01	–9.01	–9.01	0.36322
A‡	+4.26	+4.26	+4.26	+4.26	+4.26	+4.04	+4.26	+4.26	+4.26	+4.26	+4.26	0.36322
D	–11.05	–11.05	–11.05	–11.05	–11.05	–11.15	–11.05	–11.05	–11.05	–11.05	–11.05	0.36322
Between nonstructural proteins 4B and 5												
R	–16.05	–16.05	–16.05	–16.05	–16.05	–16.05	–16.05	–16.05	–16.05	–15.83	–16.05	0.37390
G‡	–13.19	–13.19	–13.19	–13.19	–13.19	–13.19	–13.19	–13.19	–13.19	–13.08	–13.19	0.37390
G	–19.54	–19.54	–19.54	–19.54	–19.54	–19.54	–19.54	–19.54	–19.54	–19.66	–19.54	0.37390

*Signal cleavage predicted scores were calculated with AnalyzeSignalase 2.03 (*14*). The table indicates only the last amino acid of the first protein and the first 2 amino acids of the following protein.
†Two-tailed Student *t* test results, indicating the probability of significance of observed differences between lineage 2 and lineage 1 strains.
‡Exact cleavage site.

ter the hydrophobicity of the NS4B protein (Ala79Thr) relative to the other strains and may have potential structural and functional implications for the viral replicase complex of which NS4B is a component (*25*).

Most amino acid differences occurred in the NS5 protein, which is associated with cytoplasmic RNA replication because it contains an RNA-dependent RNA polymerase, *S*-adenosylmethionine methyltransferase, and importin β–binding motifs (*28*). Deletions in the NS5 protein abolish replication (*29*), which suggests that amino acid substitutions may effect replication efficiency and, hence, virulence. Temperature-sensitive strains with reduced virulence for mice, isolated in Texas, also contained mutations in the NS proteins (*30*). In addition, organ tropism of strains has been associated with mutations in the NS5, NS2, and E proteins (*18*). The 2 lineage 2 strains that caused mild disease in patients (H442 and SA381/00) had several substitutions of hydrophobic to hydrophilic amino acids relative to the other 2 strains in the NS5 protein. SPU 116/89, isolated from a patient with necrotic hepatitis, had several amino acid changes that may affect its hydrophobicity and result in structural and functional changes that have implications for altered replication efficiency, tissue tropism, and pathogenicity.

Flavivirus polyproteins are cleaved either by a host signal peptidase or a viral-encoded serine protease consisting of the NS3 protease and the NS2B cofactor (NS2B-NS3) (*29*). Proteolytic processing of the C-prM and NS4A/B proteins occurs efficiently only after upstream cleavage of the signal sequence by cytoplasmic viral protease. Efficiency of signal peptidase cleavage at the NH_2 termini of prM and NS4 proteins is increased by coexpression of the viral NS2B-NS3 protease and the structural polyprotein region (*31*). Mutagenesis analysis of the signal sequence of yellow fever virus prM protein indicated that mutations that enhance cleavage by the signal peptidase almost totally suppress production of infectious virions (*31*). Signal peptidase cleavage of prM protein results in the production of membrane-anchored forms of the C protein, which may be deleterious for replication if it functions poorly as a substrate for viral protease. The signal peptidase-mediated cleavage at the NH_2 terminus of prM protein does not occur efficiently, whereas cleavage at the NH_2 terminus of the E protein does. Inadequate prM protein production in turn affects production and lowers the secretion of prM-E heterodimers. When these constructs are used in vaccination studies, a lack of immunogenicity is noted (*32*).

In the present study, all highly and less pathogenic lineage 2 strains as well as lineage 1 strains were predicted to be cleaved with the same efficiency. At the C-prM site, lineage 2 strains are cleaved slightly more efficiently than lineage 1 strains; at prM-E, the reverse is true. How these differences in cleavage efficiency affect pathogenicity is unclear and may warrant further investigation.

The high number of cases of neurologic infections in recent epidemics in the United States may be attributed to the rapid distribution of a single highly neuroinvasive strain in a highly susceptible population. The comparatively low number of WNV fever or neurologic cases reported in South Africa, despite the wide distribution of the virus

and the presence of neuroinvasive strains, may reflect inadequate surveillance and a lack of medical awareness of the disease potential of arboviruses. Moreover, the importance of WNV in South Africa may be overshadowed by the presence and effect of other diseases such as HIV/AIDS. Nevertheless, the epidemic potential and effect that WNV may have on a large population of immunocompromised HIV-infected persons necessitates improved surveillance of arbovirus infections of persons in southern Africa.

In conclusion, these full genome sequences provide insight into the molecular factors that may differentiate pathogenic from mild lineage 2 WNV strains. Mutations in the NS proteins encoding viral replication and protein cleavage mechanisms are the most likely determinants of differences in pathogenicity.

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Unexpected Occurrence of Plasmid-mediated Quinolone Resistance Determinants in Environmental *Aeromonas* spp.

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We searched for plasmid-mediated quinolone resistance determinants of the Qnr type in several water samples collected at diverse locations from the Seine River (Paris, France). The *qnrS2* genes were identified from *Aeromonas punctata* subsp. *punctata* and *A. media*. The *qnrS2* gene was located on IncU-type plasmids in both isolates, which resulted in increased MIC values of quinolones and fluoroquinolones, once they were transferred into *Escherichia coli*. The *qnrS2* gene identified in *A. punctata* was part of novel genetic structure corresponding to a mobile insertion cassette element. This identification of plasmid-mediated *qnr* genes outside *Enterobacteriaceae* underlines a possible diffusion of those resistance determinants within gram-negative rods.

Quinolones are broad-spectrum antibacterial agents used in human and veterinary medicine. Their extensive use has been associated with a rising level of quinolone resistance (*1*). The 2 main mechanisms of quinolone resistance are chromosomally encoded, either a modification of the quinolone targets with changes of DNA gyrase (*gyrA*) and/or of topoisomerase IV (*parC*) genes; or a decreased intracellular concentration due to impermeability of the membrane or to overexpression of efflux pump systems (*2*). Plasmid-mediated quinolone resistance was first identified in a *Klebsiella pneumoniae* clinical isolate from the United States (*3*). It is mediated by a 218-aa protein, Qnr (lately termed QnrA), which belongs to the pentapeptide repeat family of proteins that protects DNA from quinolone binding to topoisomerases (*4*,*5*). QnrA confers resistance to quinolones such as nalidixic acid and increases MICs of fluoroquinolones up to 32-fold in *Escherichia coli* (*6*). In addition, it enhances selection of associated chromosome-encoded quinolone resistance determinants that confer additional resistance to fluoroquinolones (*7*). The QnrA determinants have been reported worldwide in many enterobacterial species, and 6 of them are known so far (QnrA1 to QnrA6) (*8*). Other plasmid-mediated quinolone resistance determinants, QnrB (QnrB1 to QnrB10) and QnrS (QnrS1 and QnrS2), have been identified in enterobacterial species, sharing 41% and 60% amino acid identity with QnrA, respectively (*8*–*10*). The plasmid-mediated *qnr* genes have been identified so far only in *Enterobacteriaceae* (*6*,*8*). Recent findings indicated that those genes originate from environmental gram-negative bacterial species, such as *Shewanella algae,* the progenitor of the *qnrA* genes (*11*), and *Vibrio splendidus,* the progenitor of *qnrS* genes (*12*). We have shown that many *Vibrionaceae* species may harbor chromosome-encoded *qnr*-type genes (*13*).

To further evaluate the spread of plasmid-mediated resistance determinants in the environment, we have searched for those genes in water samples drawn from the Seine River in Paris, France. We identified QnrS determinants in *Aeromonas* species in uncommon genetic environments.

Materials and Methods

Water Sampling and Screening for *qnr* Genes

Water samples (40 mL each) were collected in November 2006, ≈0.2 m below the water surface, by immersion of 50-mL sterile, screw-capped tubes. Six distinct urban sites located on the Seine River in Paris were sampled: center (site 1), north (site 2), east (site 3), south (site 4), and west

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(sites 5 and 6). The Seine crosses the city of Paris (2. 4 million inhabitants) from southeast to northwest.

Samples were stored at 4°C before DNA extraction. To estimate the total number of viable bacteria, we diluted all samples in 0.85% saline solution and plated them (0.2 mL) on chromogenic URISelect4 agar (Bio-Rad, Marnes-la-Coquette, France) without any antimicrobial agent, which allowed visual differentiation of gram-negative species. After enrichment of 1 mL of water sample in 10 mL of trypticase soy (TS) broth for 48 h at 30°C and 37°C, *qnrA*-, *qnrB*- and *qnrS* genes were detected by using a multiplex PCR-based strategy (see below).

Isolation and Identification of *qnr*-Positive Isolates

To identify the *qnr*-positive isolates, 1 mL of each *qnr*-positive water sample was grown in 10 mL of TS broth containing nalidixic acid (16 mg/L) for 24–48 h at 30°C and 37°C. After dilution in 0.85% NaCl, each broth was plated (0.2 mL) on chromogenic URISelect4 agar and 5% horse-blood TS agar, both containing nalidixic acid (16 mg/L). Finally, each colony was tested by multiplex PCR for detection of *qnr* genes (see below). Further identification of *qnr*-positive isolates was performed by conventional biochemical techniques (API-20E and API-NE systems [bioMérieux, Marcy-l'Etoile, France]), and by sequencing of 16S rDNA and *gyrB* genes, as previously described (*14*,*15*).

Isolation of Total DNA and PCR Amplification

Whole-cell DNA was extracted from water samples by using QIAamp DNA Mini Kit (QIAGEN, Courtaboeuf, France), according to the manufacturer's recommendations. Whole-cell DNA from isolated colonies identified after culture was extracted by using the boiling technique, which includes a heating step at 100°C of a single colony in a volume of 100 μL of distilled water followed by centrifugation of the cell suspension.

Screening of the *qnrA*-, *qnrB*-, and *qnrS* genes in water samples and from isolated colonies was conducted by using a multiplex PCR-based technique able to amplify the known Qnr variants, as previously described (*16*). *E. coli* Lo, *K. pneumoniae* B1, and *E. coli* S7 strains were used as *qnrA*-, *qnrB*-, and *qnrS*-positive controls, respectively (*16*,*17*). PCR amplicons were sequenced on both strands (see below). The chromosome-encoded quinolone-resistance determining regions (QRDRs) of *gyrA* and *parC* genes were sequenced after PCR amplification by using whole-cell DNA of *qnr*-positive isolates, as previously described (*18*).

PCR assays were also performed by using standard conditions of amplification (*17*) to detect genes coding for the replication protein of IncU-type plasmids (*rep* gene) and the tetracycline resistance determinant (encoded by the *tetA* gene). The primers, designed for this study, are as follows: for *rep*, Rep-IncU-F (5′-CTGGCTGAAATGCTGTTGCC-3′) and Rep-IncU-R (5′-GCTTCATAGGCTTCACGCTC-3′) to give a 1,199-bp product, and for *tetA*, TetA-1 (5′-GTGAAACCCAACAGACCCC-3′) and TetA-2 (5′-TCAGCGATCGGCTCGTTGC-3′) to give a 589-bp product.

Antimicrobial Drug–Susceptibility Testing

The antimicrobial drug susceptibility of *Aeromonas* isolates was first determined by the disk diffusion technique on Mueller-Hinton (MH) agar plates according to Clinical and Laboratory Standards Institute guidelines (*19*). The disks were supplied by Bio-Rad Laboratories, and the following antimicrobial agents were tested: amoxicillin (25 μg), ticarcillin (75 μg), amoxicillin-clavulanate (20/10 μg), ticarcillin-clavulanate (75/10 μg), piperacillin (75 μg), piperacillin-tazobactam (75/10 μg), cephalothin (30 μg), cefuroxime (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), aztrem (30 μg), latamoxef (30 μg), cefepime (30 μg), imipenem (10 μg), kanamycin (30 IU), tobramycin (10 μg), gentamicin (15 μg), netilmicin (30 μg), amikacin (30 μg), nalidixic acid (10 μg), norfloxacin (5 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), tetracycline 30 (IU), fosfomycin (50 μg), rifampicin (30 μg), sulfonamide (200 μg), trimethoprim (5 μg), and colistin (50 μg).

Thus, MICs of quinolones and fluoroquinolones were determined by using the E test technique, according to the manufacturer's recommendations (AB Biodisk, Solna, Sweden). MIC breakpoints were retained for determining susceptibility and resistance ranges to nalidixic acid and ciprofloxacin were ≤8 and ≥32 mg/L and ≤1 and ≥4 mg/L, respectively, as recommended for *Enterobacteriaceae* (*19*).

Transfer of Resistance and Plasmid Analysis

Transfer of the plasmid-mediated quinolone resistance markers from *qnr*-positive *Aeromonas* isolates to *E. coli* TOP10 was attempted by using electroporation and conjugation techniques (*20*). *E. coli* TOP10 and azide-resistant *E. coli* J53 strains were recipient strains for transformation and conjugation experiments, respectively (*20*). Conjugation experiments were performed at 22°C and 37°C, as previously described (*21*). Transformants and transconjugants were selected on MH agar plates containing nalidixic acid (3 mg/L) only or containing azide (100 mg/L) plus nalidixic acid (6 mg/L), respectively. Plasmid extraction was performed from each *qnr*-positive isolate and its corresponding transformants by using the Kieser technique (*22*). Plasmid sizes were determined by electrophoresis on an agarose gel and comparison with sizes of reference plasmids (164, 66, 38, and 7 kb) of *E. coli* NCTC 50192, as pre-

viously described (*20*). All transformants were confirmed to be *qnr* positive by mulitplex PCR (see above).

Cloning experiments were performed with *Eco*RI-restricted whole-cell DNA of *Aeromonas* isolates 37 and 42, followed by ligation of DNA fragments in the *Eco*RI-site of cloning vector pBK-CMV. Recombinant plasmids were then transformed by electroporation into *E. coli* TOP10 electrocompetent cells. *E. coli* TOP10 harboring recombinant plasmids were selected on MH agar plates containing kanamycin (30 mg/L) and nalidixic acid (3 mg/L). All clones were tested as carrying *qnr* gene by multiplex PCR (see above), and cloned fragments of recombinant plasmids were sequenced by primer walking (see below).

Sequencing and Bioinformatic Analysis

PCR products, purified with a Qiaquick PCR Purification Kit (QIAGEN), and recombinant plasmids were sequenced by using an Applied Biosystems sequencer (ABI377, Foster City, CA, USA). The nucleotide sequences and the deduced protein sequences were analyzed with BLAST software (www.ncbi.nlm.nih.gov/BLAST).

Results

QnrS2 Determinant from *Aeromonas* spp.

Samples extracted from 2 of the 6 sites (sites 3 and 4) from the Seine River in autumn 2006 were PCR positive for *qnrS* genes. Sequencing of the amplicons identified a *qnrS2* gene in both cases. Further samples were therefore collected 1 week later from the same collecting sites and assessed the permanent occurrence of QnrS2- positive isolates in that river at that time. No *qnrA* and *qnrB* genes were detected.

The total number of gram-negative bacteria from water collection varied from 10^3 to 10^5 CFU per mL, depending on the sampling site, and included ≈10%–50% of isolates belonging to *Aeromonas* species (data not shown). The QnrS2-positive isolates were identified as *Aeromonas* species according to their phenotypic characterization. Strain 37 (site 4, pink colonies on URI4Select agar) and strain 42 (site 3, blue colonies on URI4Select agar) were selected as *qnrS2*-positive *Aeromonas* isolates to be further characterized. Genotypic identification was first attempted by sequencing of a ≈1,200-bp fragment of the 16S rDNA gene, but sequence analysis did not allow a precise identification. Isolate A37 was identified as *A. trota* or *A. punctata* (formerly *A. caviae*) (only 1 nt difference) and isolate A42 as *A. hydrophila* or *A. media* (only 1 nt change). Because *gyrB* sequence divergence is greater than that of 16S rDNA, phylogenetic analysis based on *gyrB* sequence (allowing more reliable identification of members of the genus *Aeromonas* [*15*]) identified isolate A37 as *A. punctata* subsp. *punctata* and isolate A39 as *A. media*.

The *Aeromonas* isolates both displayed a wild-type resistance phenotype to β-lactams with resistance to narrow-spectrum penicillins and remained susceptible to several β-lactam molecules, including broad-spectrum cephalosporins and carbapenems. *A. media* 42 was also resistant to several aminoglycosides (kanamycin, tobramycin), chloramphenicol, and tetracycline, whereas *A. punctata* 37 was fully susceptible.

Aeromonas isolates 37 and 42 were resistant to nalidixic acid (MIC >256 mg/L) and to fluoroquinolones (Table). However, *A. media* 42 exhibited higher resistance levels to fluoroquinolones, with MICs 2- to 8-fold higher than those for *A. punctata* 37 (Table). Sequence analysis of the QRDR regions of *gyrA* and *parC* genes showed that *A. punctata* 37 had 1 aa substitution, Ser83Ile in GyrA, whereas *A. media* 42 had 2 aa substitutions, Ser83Ile in GyrA and Ser80Ile in ParC, as compared with the wild-type proteins of *Aeromonas* species (*18*).

qnrS2 Gene on IncU-Plasmid Backbone

The plasmid-mediated quinolone resistance QnrS2 determinant was transferred from *Aeromonas* isolates 37 and 42 to *E. coli* TOP10 recipient strain by electrotransformation, but repeated conjugation experiments failed. Plasmid analysis identified a single 55-kb plasmid (p37) and a single 20-kb plasmid (p42) from *E. coli* transformants from *A. punctata* 37 and *A. media* 42 isolates, respectively (Figure 1). After they were transferred into *E. coli* TOP10, plasmids p37 and p42 conferred increased MICs of quinolones

Table. MICs (mg/L) of quinolones and fluoroquinolones for *Aeromonas punctata* 37, *A. media* 42, and *Escherichia coli* TOP10 harboring natural plasmids p37 and p42*

Antimicrobial agent	*A. punctata* 37	*A. media* 42	*E. coli* TOP10 with plasmids		*E. coli* TOP10
			p37	p42	
Nalidixic acid	>256	>256	4	4	1
Norfloxacin	16	64	1	1	0.03
Ofloxacin	8	>32	0.5	0.5	0.01
Ciprofloxacin	4	>32	0.12	0.25	<0.01
Moxifloxacin	4	32	0.25	0.25	<0.01
Sparfloxacin	16	>32	0.25	0.25	<0.01
Enrofloxacin	4	>32	0.5	0.5	<0.01

*Each expressing the plasmid-mediated quinolone determinant QnrS2 from *A. punctata* 37 and *A. media* 42, respectively, and *E. coli* reference strain TOP10.

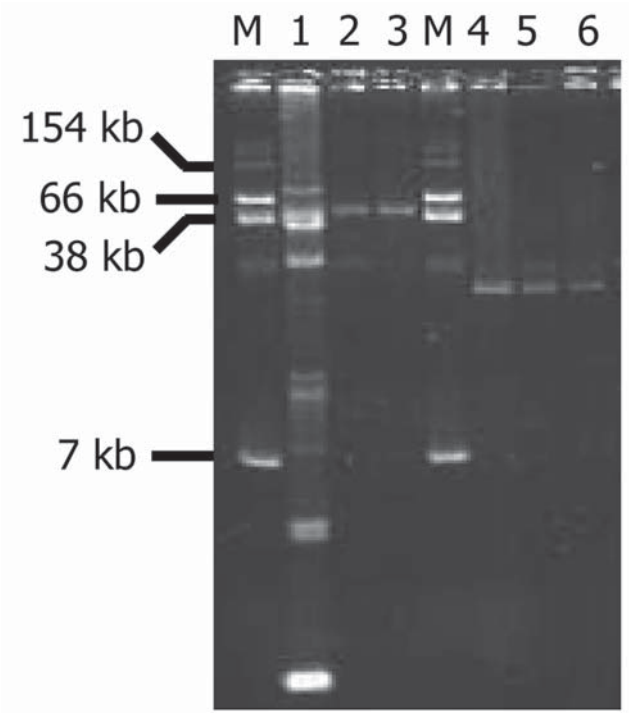

Figure 1. Plasmid DNAs from *Aeromonas punctata* 37 and *A. media* 42 and their *Escherichia coli* TOP10 transformants (TF) carrying plasmids p37 or p42. Lanes: 1, *A. punctata* 37; 2, *E. coli* TOP10/p37 TF-1; 3, *E. coli* TOP10/p37 TF-2; 4, *A. media* 42; 5, *E. coli* TOP10/p42 TF-1; 6, *E. coli* TOP10/p42 TF-2; M, *E. coli* NCTC 50192 (used as reference for plasmid sizes).

and fluoroquinolones (Table). No other antimicrobial resistance marker was carried by those natural plasmids.

Cloning of *Eco*RI-restricted DNA from whole-cell DNA of *A. punctata* 37 produced a recombinant plasmid, pAS37, containing a 19,050-bp insert that contained the *qnrS2* gene. Sequencing showed that the *qnrS2* gene was located in a plasmid-encoded genetic structure previously identified in an IncU-related plasmid, pFBAOT6 (84,748 bp), isolated from an *A. punctata* strain recovered from hospital sewage in Kendal (United Kingdom) in 1997 (*23*). This region consisted of 15 open reading frames (Figure 2). Detailed analysis of pAS37 showed that a fragment of 1,375 bp containing the *qnrS2* gene (657 bp) was inserted within the *mpR* gene coding for a putative zinc metalloprotease (MpR) (Figure 2). Sequencing of the full insert in plasmid pAS37 identified the same *rep* region as reported from the IncU-related pFBAOT6 plasmid, which indicated that p37 was a member of the IncU incompatibility group. PCR amplification that used specific primers of this *rep* gene also gave positive results from whole-cell DNA of *E. coli* (p42) transformant, indicating that p42 also belonged to IncU-type plasmid family. In addition, cloning of *Eco*RI-restricted DNA from whole-cell DNA of *A. media* 42 showed that the *qnrS2* gene was inserted into the *mpR* gene in plasmid p42. No *tetA* gene encoding resistance to tetracycline was detected by PCR in plasmids p37 and p42, whereas it was identified in plasmid pFBAOT6 (*23*).

qnrS2 Gene as Part of a Mobile Insertion Cassette

The *qnrS2* gene was identified inside a 1,375-bp structure bracketed by two 22-bp imperfect inverted repeats (4 mismatches) (Figure 2). This structure ressembled a transposon; however, no transposase encoding gene was associated with the *qnrS2* gene, the single gene identified in this genetic structure. Acquisition of that structure was likely the result of a transposition process because it was bracketed by a 5-bp duplication of the target site (CCTCC) that might be likely considered as the signature of transposition. Thus, this genetic structure defines a mobile insertion cassette (mic) containing the *qnrS2* gene instead of a transposase, as observed in insertion sequences. Those mic elements are not related to the gene cassette associated with class 1 integrons (*26*). The mic-*qnrS2* element identified here carried putative promoter sequences able to enhance the *qnrS2* expression made of a –35 box (TTCTCT) and a –10 box (TAACTT) separated by a 17-bp sequence.

Discussion

Our study identified plasmid-mediated quinolone resistance QnrS determinants from water samples collected in different sites in a Paris river. To our knowledge, this is the first identification of plasmid-mediated QnrS determinants in nonenterobacterial species. Previous studies did not identify such *qnr* genes from tested gram-negative isolates that represented *Campylobacter jejuni* (*27*), *Aeromonadaceae*, *Pseudomonadaceae*, *Xanthomonadaceae*, *Moraxellaceae*, and *Shewanellaceae* (*11*). Identification of QnrS-positive isolates at 2 collection sites in different water samples may highlight their relative persistence in the environment, at least in this area at that time.

The high-level resistance to quinolones and fluoroquinolones might be due to mutations in type II topoisomerase genes because the mutations described in type II topoisomerases in *A. media* and *A. punctata* subsp. *punctata* have already been associated with resistance in *Aeromonas* spp (*18*). QnrS2 may confer low-level resistance to quinolones, as known in *E. coli*.

The *qnrS1* gene has been identified now from several enterobacterial isolates from Japan (*9*), Germany (*28*), the United Kingdom (*29*), the United States (*25*), France (*17*), Vietnam (*30*), Taiwan (*31*,*32*), and Denmark (*33*). The *qnrS2* gene (92% amino acid identity with QnrS1) was identified from a transferable IncQ-related plasmid (pGNB2) isolated from an activated sludge bacterial community of a wastewater treatment plant in Germany (*24*) and in a single non-Typhi *Salmonella* clinical isolate from

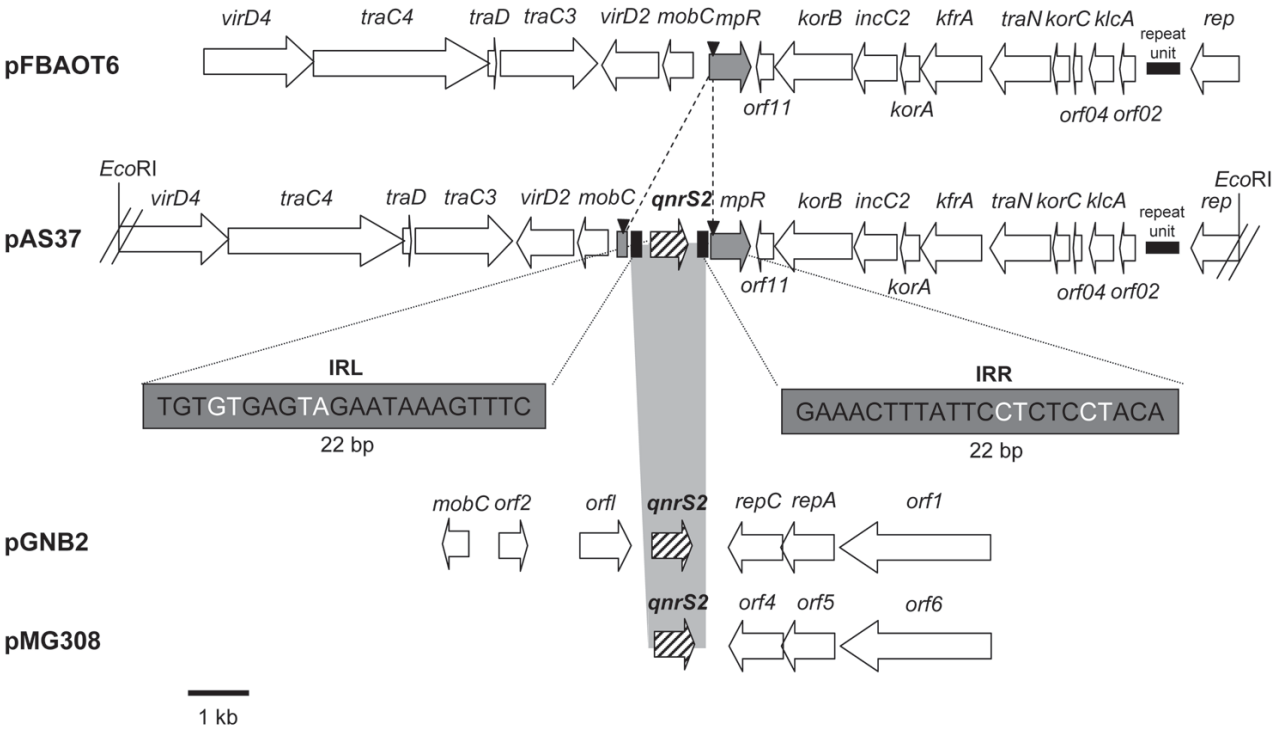

Figure 2. Genetic environments of the *qnrS2* gene in plasmid p37 from *Aeromonas punctata* 37 and comparison with related plasmid structures. Plasmid pFBAOT6 is from *A. punctata* from the United Kingdom (*23*); plasmids pGNB2 and pMG308 are from a wastewater treatment plant from Germany (unknown bacterial reservoir) (*24*) and from a non-Typhi *Salmonella* clinical isolate from the United States (*25*), respectively. Recombinant plasmid pAS37 has been obtained from our study. Open reading frames (ORFs) are indicated by horizontal arrows. The right and left inverted repeats (IRR and IRL) are indicated, and duplication sites (CCTCC) are represented by black triangles. The *Eco*RI- restriction sites that have been used for cloning experiments are indicated. The identified mobile insertion cassette element is bracketed by IRL and IRR of 22-bp size (bases in black are identical, and bases in white are different).

the United States (*25*). Identification of QnrS determinants in *Aeromonas* spp. indicates that those bacterial species may play a role as a reservoir of the *qnrS* genes in an aquatic environment, as already evidenced for *tet* genes (*34*,*35*). However, whether *Aeromonas* species are a main or an accessory reservoir of plasmid-mediated quinolone resistance determinants in regard to *Enterobacteriaceae* remains to be determined. For *Aeromonas* spp. to act as a reservoir of *qnr* genes it must be capable of acquiring these resistance genes from their progenitors (*36*) and transferring this genetic information to *Enterobacteriaceae*. QnrS2-positive plasmids p37 and p42 were not able to be transferred by conjugation to an *E. coli* host in vitro, but they were able to replicate in *E. coli*, indicating their broad host spectrum. In addition, our study demonstrated that this IncU-type plasmid-mediated *qnrS2* gene was expressed and able to confer reduced susceptibility to quinolones, at least in *E. coli*. *Aeromonas* species and IncU plasmids, which are ubiquitous in a wide range of environments, might therefore act as important vectors for transfer of plasmid-mediated quinolone resistance determinants (*23*).

As opposed to most *qnrA* and *qnrB* genes, *qnrS* genes have never been reported to be associated with *sul1*-type class 1 integrons (*6*,*8*). The *qnrS1* gene has been identified either upstream of Tn*3*-like transposon (*9*,*28*) or upstream of the insertion sequence IS*Ecl*2 (*17*,*37*). In IncQ-related plasmid pGNB2 and in pMG308 from a *Salmonella* isolate, surrounding genetic structures of the *qnrS2* genes were similar, with 2 open reading frames located immediately downstream of *qnrS2,* similar to *repC* and *repA* genes involved in plasmid replication (*24*,*25*) (Figure 2). In plasmid p37 from *A. punctata* 37, the genetic structure was different since the *qnrS2* was part of a transposon-like structure and inserted in an open reading frame coding for a zinc MpR.

We have shown that plasmid integration of the *qnrS2* gene may result from a peculiar transposition process that likely corresponds to *trans*-transposition. The *qnrS2* gene was inserted in a peculiar mic. Such mic elements have been identified rarely, e.g., mic*231*-like elements in *Bacillus cereus*, carrying in only 1 instance an antibiotic resistance gene, the *fos* gene encoding resistance to fosfomycin (*26*). This finding may indicate that mic elements might be clinically relevant and the origin of an additional gene plasticity in a bacterial species. These elements containing genetic features involved in gene dissemination (with a transposase likely acting *in-trans*) and expression may be also vehicles

for antibiotic resistance genes. This structure type may be added to the list of genetic tools at the origin of dissemination and expression of antibiotic resistance determinants.

As previously described for the spread of a carbapenemase gene (bla_{IMI-2}) in US rivers (*20*), this report underlines that the aquatic environment is an important reservoir of novel antibiotic resistance determinants. Quinolones are antimicrobial agents extensively used in aquaculture and are stable molecules in water (as opposed to β-lactams) (*38*). Thus, they may be the source of an important driving force for selection of quinolone resistance, which explains why QnrS2-positive plasmids did not possess any additional resistance determinants. Further studies might focus on the particular effect of quinolone use for inducing the *qnrS2* gene mobility because it is known those molecules may induce bacterial repair systems and antibiotic resistance gene transfer (*39*).

We have previously shown that the *qnrA* and *qnrS* genes originate from water-borne bacterial species, *S. algae* and *Vibrio splendidus*, respectively. This identification of a *qnrS* gene in another water-borne species, *Aeromonas,* further strenghens the role of water as a vehicle for spread of those resistance determinants.

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Emergence of New Norovirus Variants on Spring Cruise Ships and Prediction of Winter Epidemics

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In June 2006, reported outbreaks of norovirus on cruise ships suddenly increased; 43 outbreaks occurred on 13 vessels. All outbreaks investigated manifested person-to-person transmission. Detection of a point source was impossible because of limited investigation of initial outbreaks and data sharing. The most probable explanation for these outbreaks is increased norovirus activity in the community, which coincided with the emergence of 2 new GGII.4 variant strains in Europe and the Pacific. As in 2002, a new GGII.4 variant detected in the spring and summer corresponded with high norovirus activity in the subsequent winter. Because outbreaks on cruise ships are likely to occur when new variants circulate, an active reporting system could function as an early warning system. Internationally accepted guidelines are needed for reporting, investigating, and controlling norovirus illness on cruise ships in Europe.

Norovirus is a highly infectious causal agent of a usually mild and self-limiting acute gastroenteritis. The symptoms of vomiting and diarrhea occur after a short incubation period of 8 to 72 hours. Although norovirus can cause sporadic cases (*1*), this contagious virus is often described as a cause of outbreaks (*2*–*5*). In Europe, norovirus outbreaks are reported to the Food Borne Viruses in Europe (FBVE) network. This network maintains a Web-based surveillance database containing data reported by 13 European countries (*6*).

In June 2006, the Dutch Food and Consumer Product Safety Authority (VWA) contacted the coordinator of the FBVE network, located at the National Institute for Public Health and the Environment (RIVM) in the Netherlands. The VWA had been notified of suspected norovirus outbreaks on 3 cruise ships operating in the Netherlands in the previous month. In the same week, ProMED reported a viral gastroenteritis outbreak on a Dutch-owned cruise ship operating out of the United Kingdom (*7*). Norovirus outbreaks on cruise ships are not normally reported to national surveillance centers in Europe, but having been alerted to

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these outbreaks, these centers recognized that the number of outbreaks was unusual. In Europe, norovirus outbreaks are highly seasonal, with most outbreaks reported from October through April (*8*,*9*). Further inquiries found that passengers on several ships sailing within European waters were experiencing outbreaks of gastroenteritis. This finding resulted in a coordinated investigation between the European Centre for Disease Prevention and Control (ECDC) and the FBVE network to identify or exclude a common source of infection (*10*,*11*). The investigation was based on the hypotheses that the possible rise in reported outbreaks was 1) reporting bias resulting from media attention and active investigation of these outbreaks, 2) an actual increase specific for cruise ships by means of a common source, or 3) a reflection of actual increased norovirus activity in the community. We describe the results of data collection at the European level by an international and multidisciplinary investigation team.

Methods

Epidemiologic, virologic, and baseline data were collected from various sources. These sources included the FBVE network, ECDC, Food Safety Authorities, Early Warning Response System messages, diagnostic and reference laboratories, local health institutions, and ship owners.

Definitions

A single outbreak was defined as a cluster of at least 3 people becoming ill within 3 days of each other with symptoms of acute gastroenteritis during 1 voyage with 1 group of passengers on board a ship. A ship-level outbreak was defined as successive single outbreaks occurring on 1 ship. An outbreak was confirmed if norovirus was detected in stool samples from ≥2 patients and was considered probable if norovirus was detected in only 1 patient's sample or in ≥1 environmental samples. If descriptive clinical data suggested a viral cause but microbiologic proof for the causative agent was absent, the outbreak was considered as possibly caused by norovirus. Because norovirus outbreaks typically occur in winter, we defined a norovirus surveillance year as running from May through April of the next year to include a full winter season. Two periods were defined: off-seasonal, lasting from May through September; and seasonal, lasting from October through April of the following year.

Data Collection

Epidemiologic Data

We included outbreaks that occurred on ships sailing within Europe and that were reported between January 1 and August 1, 2006. Information describing the outbreaks was collected; the dataset is given in a footnote of the online Appendix Table (available from www.cdc.gov/EID/content/14/2/238-appT.htm). If an on-site outbreak investigation was performed, local authorities were asked to send their outbreak report to the investigation team.

Virologic Data

Environmental samples and patients' stool samples were collected and were tested at different institutions by using local protocols, primarily reverse transcription–PCR (*12*). Virus information was collected to determine the causative agent and to determine whether identical strains indicated a common source for different ships. Sequences from a specific genomic region, the polymerase region A, were analyzed, which allowed an international comparison to be made (*13*). If this analysis could not be done at a local level, stool samples, RNA, or sequence information was sent to reference laboratories.

Background Data

The FBVE database (*6*) enables analyses of combined epidemiologic and virologic data. Baseline incidence was determined by analyzing the reported outbreaks, as registered in the FBVE database in March 2007. Outbreaks with onset from May 2002 through February 2007 were selected for the analyses. Because data from surveillance data collection were incomplete and associated with delays, to assess the number of outbreaks that occurred from May through June 2006, the FBVE network conducted an email survey within the network in July 2006. In addition, outbreak data for 2006 were obtained from Australia and Hong Kong and compared with data for 2005.

Data Analysis

Epidemiologic Data

The following data were obtained retrospectively from outbreak reports: number and duration of outbreaks, overall attack rate, attack rates among passengers and crew, availability of (adequate) protocols and materials for cleaning, passenger flow during embarkation and disembarkation (possibility of contact between arriving and departing passengers), sick leave for crew, and policy for sick patients (isolation or not). For comparison of proportions, p values were calculated according to the χ^2 test and Fisher exact test, if appropriate. Available epidemic curves were used to determine whether a point source infection was indicated through log-normal distribution (*14*), with patients clustering within 1.5× the incubation period range (CDC Manual, available from www.cdc.gov/health/botulism.htm; the manual was adapted for general epicurves, available from www.epi.state.nc.us/epi/gcdc/manual/Epicurves.pdf). Data were analyzed by using PEPI 4.0 (Programs for Epidemiologists; Sagebrush Press, Salt Lake City, UT, USA).

Virologic Data

Nucleotide sequences were aligned through Bionumerics software version 4.6 (Bionumerics package, Applied Maths, Ghent, Belgium). These sequences were then compared with consensus sequences by using a publicly available, Web-based, quick-typing tool (available from www.rivm.nl/bnwww).

Background Data

The differences between the off-seasonal number of reported outbreaks in 2006 (x_1) and the off-seasonal numbers of reported outbreaks in previous years (x_{2-5}) were compared; the difference was significant according to the following equation:

$$\frac{N(x_1) - N(x_2)}{\sqrt{Nx_1 + Nx_2}} > 1.96$$

In addition, we compared the numbers of off-seasonal, seasonal, and cruise ship outbreaks in 2006 and 2007 with the numbers from previous years, from the FBVE database. Annual seasonal numbers of reported outbreaks were ranked to assess Spearman rank correlation coefficients. Poisson regression analysis was performed to determine whether the numbers of off-seasonal outbreaks were independent from the numbers of cruise-related outbreaks. In the FBVE database, outbreaks that occur on ships are reported in the category aircraft/ship/train/bus; analysis of cruise ship outbreaks used this category, which led to some misclassification of cruise ship outbreaks. Some additional information on setting is given in free text fields and, if available, was used to reduce misclassification. Data were analyzed by using SAS 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

During the study period, 43 single outbreaks were reported from 13 vessels: 14 (33%) of these were confirmed, 2 (5%) were considered probable, and 27 (63%) were considered possible norovirus outbreaks. For ship-level outbreaks, norovirus infection was confirmed for 10 (77%) vessels, 1 (8%) ship had probable norovirus infections, and 2 ships (15%) had possible norovirus infections.

Epidemiologic Data

Of the 43 outbreaks, 1 occurred in January 2006; all others occurred from April 24 through July 21, with only a 2-week outbreak-free period (Figure 1). Three outbreaks on 3 ships occurred during the season; 40 outbreaks on 10 ships occurred during off-season months. The online Appendix Table shows available epidemiologic and virologic data for each cruise ship that had confirmed or probable norovirus outbreaks. Overall attack rates varied from <1% to 41%. The highest attack rates were 48% for passengers and 19% for crew members. Ships 10 and 13, which were ferries, reported the lowest overall attack rates and higher attack rates for crew than for passengers (p = 0.021 for ship 10; p = 0.064 for ship 13).

Source of the Outbreaks

For 3 ships, the epidemic curve of the initial outbreak was available. One of these indicated a point source, which could not be identified during the outbreak investigation. For 7 of the 13 ships, at least 15 food suppliers were identified. Of these, 13 suppliers delivered products to 1 ship and 2 suppliers delivered to multiple ships (online Appendix Table). A common food source could not be identified for all ships. For 6 ships, no information on the food supplier was available.

A retrospective cohort study performed on 1 ship and case-control studies on 2 other ships could not find any evidence of a point source. Person-to-person spread was believed to be the predominant route of transmission as shown in these analytical studies and in another 6 descriptive reports.

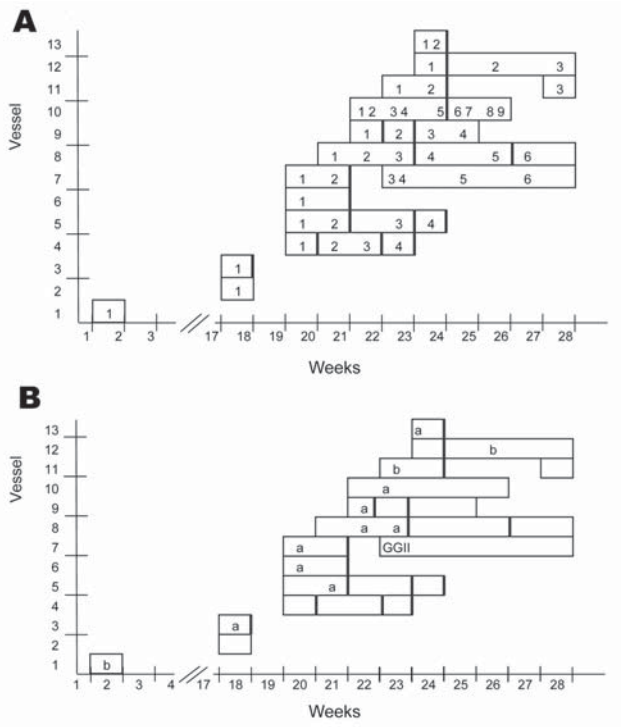

Figure 1. Number of outbreaks (A) and causative genotypes (B) for cruise-related outbreaks of norovirus for each ship from January through July 2006. Data were derived from multiple sources, active case finding, and case reports. Vertical black lines indicate marker for reported cleaning activities with extra intensity. a, GGII.4–2006a; b, GGII.4–2006b; GGII, GGII but variant unknown

Risk Factors for Multiple Outbreaks

Reports from local investigation teams were available for 7 of 9 ships that experienced multiple outbreaks and for 2 of 4 ships that experienced only 1 outbreak. These low numbers, including missing values, did not enable analysis to identify risk factors for multiple outbreaks. Descriptive information indicated the following risk factors: possible contact between boarding and disembarking passenger groups and cleaning with inappropriate materials for norovirus elimination during the first outbreak.

Virologic Results

The norovirus sequences, detected in fecal or environmental samples, were all of the GGII.4 genotype but in 2 distinct new lineages, designated GGII.4–2006a and GGII.4–2006b (Appendix Table and Figure 1) (*15*). Samples taken from 8 (73%) and 3 (27%) of 11 ships were identified with the GGII.4–2006a and GGII.4–2006b variant, respectively.

For 3 ships, the norovirus strains obtained from environmental samples were genetically identical to those obtained from patient samples. Positive environmental samples were derived from contact surfaces, which implied that person-to-person transmission through aerosols and contact with contaminated surfaces was possible. For 1 ship, samples of raspberries and tap water taken during the outbreak were found to be contaminated with norovirus. Whether the contamination was the source of the outbreak or resulted from contact with patients affected in the outbreak could not be determined.

Analysis of Background Norovirus Activity

FBVE Database

From May 1, 2002, through February 28, 2007, a total of 9,425 norovirus outbreaks were reported to the FBVE network. A total of 2,480 outbreaks occurred during the norovirus surveillance year 2006–2007. For 8 of the countries, analysis of the number of off-season outbreaks from 2002 through 2006 was possible. A combined total of 137 outbreaks were reported by these countries during the 2006 off season. This number is higher than that for the same months in 2003 (n = 68, significant), 2004 (n = 127, not significant) and 2005 (n = 132, not significant) but lower than that for 2002 (n = 383, significant) when norovirus activity was very high. However, reporting for the year 2004–2005 has been considerably delayed (median 157 days, range 4–616). Since data were derived from the database on March 14, 2007, the numbers of reported outbreaks in the surveillance database are still increasing. An average of 5 aircraft/ship/train/bus-related outbreaks per year is reported in the FBVE database (Figure 2). The Spearman rank correlation coefficient was significant between the number of outbreaks in this category and the number of off-season outbreaks (R = 0.97; p = 0.0048). Poisson regression analysis showed that the annual number of off-season cruise ship outbreaks associated strongly with the total annual numbers of outbreaks in the subsequent season (p = 0.0078) as well as the same off-season (p<0.001).

In the norovirus surveillance year 2005–2006, GGII.4 strains were the predominant type identified (79%). The 2 new lineages within the GGII.4 genotype were first detected between January and March and displaced the resident GGII.4–2004 strains (Figure 2).

Survey

Of 13 collaborating countries in the FBVE network, 11 reported higher norovirus activity at the time of the ongoing cruise ship outbreaks. Australia and Hong Kong (*16*) experienced higher norovirus activity from January through June 2006, compared to the same period in 2005: a more than 10-fold increase was reported in Adelaide (147 for 2006, 117 of which occurred out of season from January through July), and Hong Kong reported 99 outbreaks from January through June 2006 compared with 46 outbreaks in 2005. In Australia and Hong Kong, outbreaks were associated with the new lineages 2006a and 2006b, respectively.

Discussion

An unexpectedly high number of outbreaks on cruise ships in European waters in the spring and summer of 2006 triggered this investigation. Concomitant with this increase of norovirus outbreaks on cruise ships, we noted a marked increase in norovirus activity in the general population. The overall increase in norovirus activity in summer 2006 coincided with the emergence of 2 new norovirus GGII.4

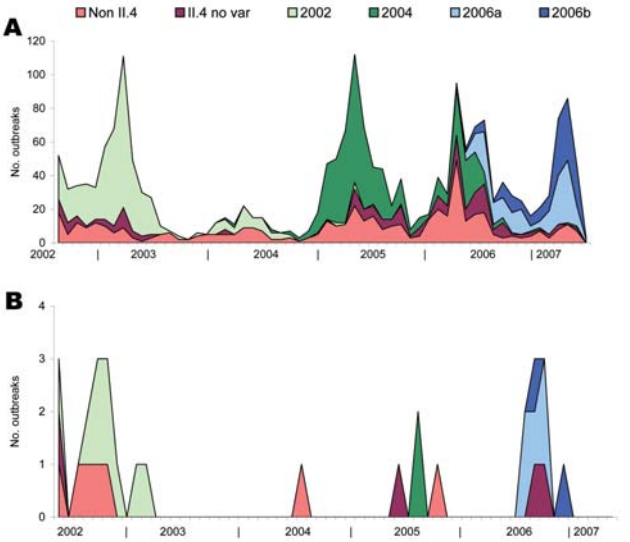

Figure 2. Cumulative outbreak data over time (2002–2007) from Food Borne Viruses in Europe network database. The total number of reported outbreaks (A) contrasted with the reported ship-related outbreaks (B). Both show norovirus strains involved.

strains and was followed by a higher number of outbreaks than usual in winter in 2006–2007 (*17*). Reporting of cruise ship related norovirus outbreaks may have been influenced by heightened attention from the media; however, the increase in reported land-based outbreaks is indicative of a real increase.

A similar situation occurred in the spring and summer of 2002, when a new variant of the GGII.4 strain emerged globally. This variant was found on cruise ships through the US Vessel Sanitation Program and in nursing homes and hospitals through the FBVE network (*18–20*). Retrospective analysis of 5 years of surveillance data from the FBVE network also showed a correlation between the number of off-season outbreaks on cruise ships and higher norovirus activity in the subsequent winter season. This recurring situation implies that cruises are possibly an early indicator for increased norovirus activity in the community because they are highly susceptible to norovirus outbreaks and mostly sail during warmer months of the year. A prospective and active surveillance program could demonstrate the validity of cruise ship outbreak incidence as a predictor of norovirus activity for the next season. After the first infection is introduced in this closed setting, an outbreak is likely to occur through person-to-person transmission (*9*). With the regular changing of passenger groups, the noroviruses on board are able to repeatedly infect a new susceptible population (*21*). Exhaustive control measures may not always be sufficient to eliminate the virus; a striking example is a positive environmental swab from a handle of a hand sanitation container, which was used before entering a restaurant. To get a better understanding of the epidemiology of noroviruses aboard cruise ships, we need a definition for a single outbreak that is more stringent than the one we used. That will only be possible with some level of routine monitoring of illnesses.

Our results could neither indicate nor exclude a point source or a common link through food or water. Information on food supply was incomplete and difficult to obtain. Separation of a potential point source from person to person or environmental transmission can optimally be investigated during a ship's initial outbreak. This investigation was only conducted on 1 ship. That 2 different lineages of GGII.4 norovirus were involved provided some evidence that a common source for all ships was unlikely (Figure 1). That a common source was unlikely was further supported by the background data showing the emergence of the same viruses coinciding with increased reporting of outbreaks from all kinds of settings across the network (Figure 2).

Attack rates for crew and passengers differed. Attack rates for the crew were mostly lower than rates for passengers, which may have been due to short-term immunity, possibly acquired during successive outbreaks over long periods (*22*,*23*). However, reporting bias is possible because crew members may be reluctant to admit to being ill (*24*). The only 2 ships in which attack rates were higher for the crew were the 2 ferries. This finding is likely due to an underestimation of number of ill passengers, because their stay on board is shorter than the average incubation period. This explanation is supported by the fact that some ferry passengers were coincidentally discovered to have been ill during their return trip 2 days later.

Patient samples are needed to confirm the causative agent of the gastroenteritis outbreaks and to analyze the genetic sequence of viruses. Typing the norovirus strain will help show whether the outbreak is likely the result of reintroduction of the virus through a person. Person-to-person transmission is likely when community norovirus prevalence is high (*25*) and is a situation that shipping companies may be unable to prevent even if they are adhering to good cleaning procedures. Unfortunately, reintroduction of the virus through a new strain could not be determined in our study for 2 reasons. First, patient sampling when passengers fall ill on cruise ships in Europe is not standardized. Virus genotyping data for subsequent outbreaks were acquired from only 1 ship, where they were identical. Second, our data may have been insufficient to discriminate between GGII.4 variants and to determine sequence diversity. New variant strains of GGII.4 emerged over a vast geographic region within a short period of time, resulting in the finding of similar or identical sequences in outbreak strains collected throughout Europe. The level of genome analysis needed to enable discrimination between individual outbreak strains remains to be determined (*26*).

International outbreak surveillance can 1) provide background data on baseline activity of the virus and circulating strains and 2) facilitate tracing of foodborne sources, especially in the case of diffuse outbreaks that may result from centralized production and wide geographic distribution of products. At times of unusual numbers of outbreaks, additional active data collection helps compensate for underreporting, reporting delays, and helps elucidate export routes of foods. In the situation described here, thorough outbreak investigation was complicated as a result of continuation of trips through different countries during the course of outbreaks. This problem is a point of concern during potential common-source outbreaks, in which early detection of the source is crucial; this matter was considered by the ECDC, which launched an initiative for measures in Europe (*27*).

Gastroenteritis outbreaks on cruise ships may seem a luxury problem. However, at times of increased norovirus activity they are likely to occur and to receive much media attention. Higher norovirus activity appeared to coincide with emergence of new variant GGII.4 strains and with a higher number of cruise-related outbreaks in the preceding spring and summer. Cruise ship holidays create an envi-

ronment in which norovirus is easily spread and outbreaks readily occur. Therefore, a reporting system for cruise ship–related outbreaks of gastroenteritis including virus detection and typing may function as an early warning system for high-epidemic winters. Such a system may enable a quick response and minimize negative effects of increased norovirus activity.

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Cost-effectiveness of Human Papillomavirus Vaccination in the United States

Harrell W. Chesson,* Donatus U. Ekwueme,* Mona Saraiya,* and Lauri E. Markowitz*

We describe a simplified model, based on the current economic and health effects of human papillomavirus (HPV), to estimate the cost-effectiveness of HPV vaccination of 12-year-old girls in the United States. Under base-case parameter values, the estimated cost per quality-adjusted life year gained by vaccination in the context of current cervical cancer screening practices in the United States ranged from $3,906 to $14,723 (2005 US dollars), depending on factors such as whether herd immunity effects were assumed; the types of HPV targeted by the vaccine; and whether the benefits of preventing anal, vaginal, vulvar, and oropharyngeal cancers were included. The results of our simplified model were consistent with published studies based on more complex models when key assumptions were similar. This consistency is reassuring because models of varying complexity will be essential tools for policy makers in the development of optimal HPV vaccination strategies.

In 2000, the Institute of Medicine (IOM) published a report listing 26 candidate vaccines that potentially could be developed and licensed in the first 2 decades of the 21st century (*1*). Included in this list was a candidate vaccine for human papillomavirus (HPV), a virus that can cause cervical and other anogenital cancers, genital warts, and other adverse health outcomes (*1*–*5*). For example, in the United States, HPV types 16 and 18 cause ≈70% of cervical cancer, 80% of anal cancer, and 30% of vaginal and vulvar cancers (*2*–*5*). Furthermore, HPV types 6 and 11 cause >90% of cases of anogenital warts (*5*,*6*). The economic costs of HPV-related genital warts and cervical disease, including screening to prevent cervical cancer, are estimated to be at least $4 billion annually in the United States (*7*,*8*).

In June 2006, the US Food and Drug Administration approved a quadrivalent (HPV 6, 11, 16, 18) vaccine (Gardasil, manufactured by Merck & Co., Inc. [Whitehouse Station, NJ, USA]) for use in girls and women 9–26 years of age (*5*). The efficacy of this vaccine is almost 100% if given to young women before sexual exposure (*3*,*5*,*9*). Also in June 2006, the US Advisory Committee on Immunization Practices recommended routine HPV vaccination for girls 11–12 years of age (*3*). The vaccine series can be initiated in girls as young as 9 years, and catch-up vaccination is recommended for girls and young women of ages 13–26 years who have not received the HPV vaccine previously or who have not completed the full vaccine series (*3*).

In anticipation of the approval of new HPV vaccines, several studies have been conducted to estimate the potential cost-effectiveness of HPV vaccination in the United States in terms of the cost per quality-adjusted life year (QALY) saved (*1*,*9*–*13*). With 1 exception (*1*), these studies applied a Markov model, a decision model, a dynamic transmission model, or a combination thereof (see Dasbach et al. [*14*] for a review of HPV models). To complement these existing studies, we developed a simplified model to estimate the cost-effectiveness of adding HPV vaccination of 12-year-old girls to existing cervical cancer screening practices in the United States. Our approach was similar to that used by IOM (*1*) in that we estimated the potential benefits of HPV vaccination based on current, age-specific incidence rates of HPV-related outcomes. Additionally, our analysis extended the IOM approach to reflect a more current understanding of the vaccine's characteristics and to include the potential benefits of preventing HPV-related anal, vaginal, vulvar, and oropharyngeal cancers.

Methods

Similar to the IOM approach, we used spreadsheet software to build an incidence-based model of the health and economic effects of HPV-related health outcomes in

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the absence of HPV vaccination. We then examined how these effects might change over time because of HPV vaccination, based on factors such as the number of 12-year-old girls vaccinated each year and vaccine efficacy. We adopted a societal perspective and included all direct medical costs (2005 US$) and benefits regardless of who incurred the costs or received the benefits (*15*,*16*). The study question we addressed was "What is the cost per QALY gained by adding vaccination of 12-year-old girls to existing cervical cancer screening practices in the United States?"

Population Model

A hypothetical population of persons 12–99 years of age was created as follows. First, the number of 12-year-old girls was based on recent sex-specific population estimates (*17*). The number of 13-year-old girls was calculated as the product of the number of 12-year-olds and the probability of survival (using recent mortality data) from age 12 years to age 13 years. The number of 14-year-old girls and the number of persons of all subsequent ages through 99 years were calculated in an analogous manner. We assumed that the number of 12-year-olds each year was constant over time so that the age distribution of the population was constant over time as well.

Vaccine Coverage, Efficacy, and Costs

We assumed the HPV vaccine would be administered to 12-year-old girls starting in year 1 and continuing through year 100. We assumed that vaccinated girls would receive the full vaccine series (3 doses) before age 13 years. Vaccination coverage (the percentage of 12-year-old girls vaccinated) was assumed to increase linearly for the first 5 years to 70% and to remain at 70% thereafter (*9*). Vaccination efficacy was assumed to be 100%, on the basis of trials showing high efficacy of prophylactic HPV vaccines against persistent infection and vaccine type–specific cervical intraepithelial neoplasia (CIN) grades 2 and 3 (*3*,*18–21*). The duration of vaccine protection was assumed to be lifelong, and the cost of vaccination was set to $360 per series (*9*).

Adverse Health Outcomes Averted by Vaccination

We examined the following HPV-related health outcomes: cervical cancer; CIN grades 1, 2, and 3; genital warts; and, in some analyses, anal, vaginal, vulvar, and selected oropharyngeal cancers. The age-specific incidence rates of the HPV-related health outcomes were used to estimate the potential reduction in these outcomes that could be obtained through vaccination.

Age-specific cancer incidence rates were derived from 2003 population-based cancer registries that participate in the Centers for Disease Control and Prevention's National Program of Cancer Registries (NPCR) and the National Cancer Institute's Surveillance, Epidemiology, and End Results Program (SEER) (*22*,*23*). Together, the 2 cancer registries covered ≈96% of the US population in 2003 (*22*). The cancer incidence rates we applied were conservative because we included only certain morphology (histology) codes, which limited cervical cancers to cervical carcinomas (squamous cell, adenocarcinoma, adenosquamous, and other carcinoma) and which limited all other noncervical cancers to squamous cell carcinomas only (*24*). We did not include in situ cancers from the cancer registries. We limited oropharyngeal cancers to selected sites most commonly associated with HPV (base of tongue, tonsillar, and other oropharyngeal sites as described in the online Technical Appendix, available from www.cdc.gov/EID/content/14/2/244-Techapp.pdf) (*24*).

Age-specific incidence rates of CIN grades 1, 2, and 3, and prevalence rates of genital warts were based on estimates obtained from the literature (*25*,*26*). We used prevalence estimates for genital warts because age-specific incidence estimates were not available (online Technical Appendix).

Cervical Cancer Screening

The incidence rates of CIN and cervical cancers that we applied in our model are those that arise in the context of current cervical cancer screening and sexually transmitted disease prevention activities in the United States. Because these prevention activities are reflected in the incidence rates of CIN and cervical cancer that we applied in our model, no information about these prevention activities (e.g., coverage and frequency of cervical cancer screening) was required in our analysis.

Costs Averted and QALYs Saved by Vaccination

The cervical cancer treatment costs averted by vaccination were calculated each year by multiplying the age-specific number of cervical cancer cases averted by the vaccine in that year by the estimated cost per case of cervical cancer (online Technical Appendix). The number of QALYs saved by preventing cervical cancer was calculated for each year by multiplying the age-specific number of cervical cancer cases averted by the vaccine in that year by the estimated age-specific number of QALYs lost per case of cervical cancer (online Technical Appendix). For other health outcomes (other cancers, CIN 1, CIN 2, CIN 3, and genital warts), the treatment costs averted and QALYs saved by vaccination were estimated in an analogous manner.

Age-specific Estimates of Direct Medical Costs and QALYs Lost per Adverse Health Outcome

The estimated direct medical cost per case of cervical cancer and other HPV-related health outcomes was based on several sources (*7*,*10*,*12*,*26–35*). The age-specific estimates

of the discounted number of QALYs lost per case of an HPV-related heath outcome (e.g., cervical cancer) were based on published estimates of the quality of life without these adverse health outcomes (*36*) and the estimated reduction in quality of life associated with the HPV-related health outcome (*1*,*10*,*12*,*37*) (online Technical Appendix).

Incremental Cost per QALY Gained

Vaccination costs, averted treatment costs, and the number of QALYs saved were calculated for each year over a 100-year period, discounted to present value by using an annual discount rate of 3% (*9*). The incremental cost per QALY gained by adding vaccination to existing cervical cancer screening was calculated as the net cost of vaccination divided by the number of QALYs gained by adding vaccination to existing screening, where the net cost of vaccination is the cost of vaccination minus the treatment costs averted by adding vaccination to existing screening (*16*).

Herd Immunity Scenario

To examine how the estimated cost-effectiveness of vaccination might change if the benefits of herd immunity were included, we assumed an additional effect of the vaccine on nonvaccinated persons, including a reduction in genital warts in men. The online Technical Appendix provides details of the methods and assumptions used to estimate these additional benefits.

Cohort Model

To make our results more comparable to Markov models of an age cohort, we modified our population model to examine the benefits of vaccination of a single cohort of 12-year-old girls over time. Vaccination costs were incurred in the first year only, and the benefits of vaccinating the 12-year-old cohort were calculated through age 99 years. Because Markov models of age cohorts typically do not include transmission dynamics, we did not consider the potential benefits of herd immunity in the cohort model.

Base Case Analyses

Using base-case parameter values (see online Technical Appendix), we estimated the cost-effectiveness of HPV vaccination by using 12 variations of the model. These 12 variations consisted of 4 permutations (including vs. excluding the noncervical cancers and including vs. excluding the benefits of preventing HPV types 6 and 11) of 3 model versions (population model with and without herd immunity, cohort model without herd immunity).

Sensitivity Analyses

We performed sensitivity analyses to examine how changes in the base-case parameter values influenced the estimated cost-effectiveness of vaccination. We first examined how the cost-effectiveness estimates of the population model's herd immunity scenario changed when assumptions about the degree of the effect of herd immunity were changed. The remainder of the sensitivity analyses focused on the population model of the quadrivalent HPV vaccine without the adjustment for herd immunity.

We performed 1-way sensitivity analyses in which we varied 1 set of parameter values while holding other parameters at their base-case values. The parameters we varied included the cost of the vaccine series ($300, $490), vaccine efficacy (95%, 99%), the cost per case of all HPV-related health outcomes (±25% of their base-case values); the discount rate (0%, 5%); the time horizon over which vaccination costs and benefits were assumed to accrue (25 years, 50 years); the incidence rates of health outcomes (±25% of their base-case values for CIN 1, CIN 2, CIN 3, and genital warts, and the lower and upper bound ranges of the 95% confidence interval from the NPCR and SEER data for cancers); the percentage of each health outcome attributable to HPV vaccine types (±20% of their base-case values); and the number of lost QALYs associated with each HPV outcome. We manipulated the last number by varying the reduction in quality of life (±50% of the base-case values) associated with all HPV-related health outcomes and by varying the stage-specific survival probabilities for HPV-related cancers (±2 standard errors). We also performed multiway sensitivity analyses by varying ≥2 sets of these parameter values simultaneously.

The parameters that were varied in the sensitivity analyses comprised almost all of the parameters in the model. Exceptions included duration of vaccine protection (which is difficult to modify in our model without sacrificing the simplicity of our approach), vaccine coverage (which does not affect our results except when herd immunity is assumed), and other parameters such as age-specific death rates, which are not subject to considerable uncertainty.

Comparison to Previous Cost-Effectiveness Studies

We compared our results with previously published estimates of the cost-effectiveness of HPV vaccination. To do so, we modified the parameter inputs to match as closely as possible several key attributes of the models applied in these previous studies (online Technical Appendix).

Results

Under base-case parameter values, the estimated cost per QALY gained by adding vaccination of 12-year-old girls to existing cervical cancer screening was $3,906–$14,723, depending on the type of model applied (cohort vs. population), whether herd immunity effects were assumed, the types of HPV targeted by the vaccine (bivalent vs. quadrivalent), and whether the benefits of preventing other cancers in addition to cervical cancer were included

(Table 1). If all other factors were equal, the estimated cost per QALY gained by vaccination was lower when herd immunity effects were assumed, when protection against HPV types 6 and 11 (rather than just HPV types 16 and 18) was included, and when the benefits of preventing other cancers in addition to cervical cancer were included.

Prevention of HPV-related health outcomes resulted in averted treatment costs and QALYs saved. For example, in the population model of the quadrivalent vaccine (when herd immunity benefits and the benefits of preventing cancers other than cervical were excluded), reductions in CIN, cervical cancer, and genital warts accounted for ≈70%, 19%, and 12% of the averted costs, respectively, and ≈33%, 54%, and 13% of the saved QALYs, respectively.

Sensitivity Analyses

The cost-effectiveness ratios did not change substantially when we modified the assumptions in the population model about the effect of herd immunity. When varying the effect of herd immunity, the cost per QALY gained by vaccination was $3,423–$7,596 for the quadrivalent vaccine and $8,549–$12,354 for the bivalent vaccine, when the benefits of preventing cancers other than cervical were excluded (results not shown).

In the 1-way sensitivity analyses of the population model (excluding assumed herd immunity effects), the discount rate and the time horizon had the greatest effect on the estimated cost per QALY gained (Table 2). When the discount rate was varied from 0% to 5%, the cost per QALY gained ranged from $675 to $24,901 (and from <$0 to $21,966 when other cancers in addition to cervical cancer were excluded). When the time horizon was varied from 25 to 50 years (rather than the base-case value of 100 years), the cost per QALY gained ranged from $21,600 to $81,786 (and from $19,943 to $81,398 when other cancers in addition to cervical cancer were included). Changes in the other sets of parameter values (such as costs and QALYs associated with HPV-related health outcomes) also affected the results, but to a lesser degree than changes in the discount rate and time horizon (Table 2). In the multiway sensitivity analyses, simultaneously changing 2 sets of parameter values resulted in estimated costs per QALY gained of <$0 to $4,606 when parameter values more favorable to vaccination were applied and estimated costs per QALY gained of $17,825 to $36,503 when parameter values less favorable to vaccination were applied (Table 3).

In the best and worst case scenarios (when all 6 selected sets of parameters were set to values more favorable and less favorable to vaccination, respectively), the cost per QALY gained was <$0 and $122,976, respectively (<$0 and $115,896 when including other cancers in addition to cervical cancer) (Table 3). However, much of the variation in the best and worst case scenarios was attributable to changes in the discount rate and the time horizon. For example, when the worst case scenario was modified to include a discount rate of 3% (rather than 5%), the estimated cost per QALY gained (when the benefits of preventing cancers other than cervical were excluded) was ≈$75,000 when applying a 50-year time horizon and $41,000 when applying a 100-year time horizon (results not shown).

Comparison with Previous Cost-Effectiveness Studies

Estimates from the simplified model were quite consistent with published estimates (Table 4). The absolute difference between the estimated cost per QALY gained by vaccination as estimated by our simplified model and as estimated by the more complex models did not exceed $4,000.

Discussion

We developed a simple model to estimate the cost-effectiveness of HPV vaccination in the context of current cervical cancer screening in the United States. We found that the cost per QALY gained by adding routine vaccination of 12-year-old girls to existing screening practices ranged from $3,906 to $14,723 under base-case parameter values (depending on the model version we applied) and ranged from <$0 (cost-saving) to $122,976 in the sensitivity analyses when several key parameter values were varied. Our results were consistent with results of published studies based on more complex models, particularly when key assumptions (e.g., vaccine duration, efficacy, and cost) were similar.

Table 1. Estimated cost per QALY gained by adding routine HPV vaccination of 12-y-old girls to existing cervical cancer screening in the United States*

Parameter	Population model		Cohort model;
	No herd immunity, $US	Herd immunity, $US	no herd immunity, $US
Excluding anal, vaginal, vulvar, and oropharyngeal cancers			
Vaccine targets HPV types 6,11,16,18	10,294	5,336	8,593
Vaccine targets HPV types 16,18	14,723	10,318	12,562
Including anal, vaginal, vulvar, and orophayngeal cancers†			
Vaccine targets HPV types 6,11,16,18	8,137	3,906	6,430
Vaccine targets HPV types 16,18	11,602	7,848	9,471

*When applying base-case parameter values to 12 model variations. QALY, quality-adjusted life year; HPV, human papillomavirus.
†The oropharyngeal cancer sites we included were base of tongue, tonsillar, and other sites as described in the online Technical Appendix (available from www.cdc.gov/EID/content/14/2/244-Techapp.pdf).

Table 2. One-way sensitivity analyses: estimated cost per QALY gained by adding routine vaccination of 12-y-old girls to existing cervical cancer screening in the United States*

Parameter or parameter set varied	Values applied in sensitivity analysis	Cost/QALY gained	
		Excluding anal, vaginal, vulvar, oropharyngeal cancers, $US	Including anal, vaginal, vulvar, oropharyngeal cancers, $US
None	NA	10,294	8,137
Vaccine cost per series (base case = $360)	$300, $490	5,811–20,009	4,237–16,587
Vaccine efficacy (base case = 100%)	95%, 99%	10,566–11,710	8,374–9,369
Cost of cervical cancer, CIN 1–CIN 3, genital warts*	Base case ±25%	6,142–14,446	4,332–11,953
Reduction in quality of life due to HPV-related health outcomes	Base case ±50%†	7,720–15,519	6,141–12,135
Incidence rates of cervical cancer, CIN 1–CIN 3, genital warts‡	Base case ±25%†	6,999–16,333	5,181–13,379
% of health outcomes attributable to HPV vaccine types	Base case ±20%	6,014–17,020	4,400–13,987
Discount rate (base case = 3%)	0%, 5%	675–24,901	<0–21,966
Time horizon (base case = 100 y)	25 y, 50 y	21,600–81,786	19,943–81,398

*When key parameter values were varied in the population model of quadrivalent HPV vaccine (excluding herd immunity). QALY, quality-adjusted life year; HPV, human papillomavirus; NA, not applicable; CIN, cervical intraepithelial neoplasia.
†See text and online Technical Appendix (available from www.cdc.gov/EID/content/14/2/244-Techapp.pdf) for details.
‡And, when applicable, anal, vaginal, vulvar, and oropharyngeal cancers.

The simplicity of our approach offers advantages and disadvantages. The main advantage is that it requires substantially fewer assumptions than the more complex Markov and transmission models. For example, there is no need to model the probability of HPV acquisition, the possible progression from HPV infection to disease, the mixing of sex partners, the probability of HPV transmission, and so forth. There also is no need to model cervical cancer screening and sexually transmitted disease prevention activities because these activities are reflected in the incidence rates of HPV-related health outcomes that we applied.

Because we do not model cervical cancer screening directly, however, we are unable to use our model to examine how changes in cervical cancer–screening strategies can affect the cost-effectiveness of HPV vaccination, and vice versa. For example, HPV vaccination is expected to reduce the positive predictive value of abnormal Papanicolaou (Pap) test results (*38*). However, our analysis did not include the loss in quality of life attributable to the initial distress associated with receiving an abnormal Pap result (*39*), regardless of whether it is a false positive. This omission of the lost QALYs due to abnormal Pap test results underestimates the benefits of HPV vaccination because vaccination is expected to offer moderate reductions in the number of abnormal Pap results overall (*38*,*40*). Future changes in screening strategies, such as delayed screening, could also possibly improve the cost-effectiveness of HPV vaccination (*12*).

Another disadvantage of our approach is that it offers only a rough approximation of the cost-effectiveness of HPV

Table 3. Multiway sensitivity analyses: estimated cost per QALY gained by adding routine vaccination of 12-y-old girls to existing cervical cancer screening in the United States*†

Parameter or parameter set varied	Cost per QALY gained	
	Excluding anal, vaginal, vulvar cancers, $US.	Including anal, vaginal, vulvar cancers, $US
Higher cost per case and larger reduction in quality of life for all HPV-related health outcomes	4,606	3,262
Lower cost per case and smaller reduction in quality of life for all HPV-related health outcomes	21,779	17,825
Discount rate = 0%; time horizon = 100 y	675	<0
Discount rate = 5%; time horizon = 50 y	36,503	34,539
Higher percentage of health outcomes attributable to HPV vaccine types; higher incidence of HPV-related health outcomes	3,815	1,882
Lower percentage of health outcomes attributable to HPV vaccine types; lower incidence of HPV-related health outcomes	24,250	20,265
All variables above (best-case scenario)	<0	<0
All variables above (worst-case scenario)	122,976	115,896

*When key parameter values were simultaneously varied in the population model of quadrivalent HPV vaccine (excluding herd immunity). QALY, quality-adjusted life year; HPV, human papillomavirus;
†The lower and upper bound ranges were the same as described in the1-way sensitivity analyses, except for the time horizon, which was varied from 50 y to 100 y.

Table 4. Summary of previously published models and estimates of the cost per QALY gained by adding routine HPV vaccination of 12-y-old girls to existing cervical cancer screening in the United States*†

Variable	Goldie et al. 2004 (*10*)	Sanders and Taira 2003 (*11*)	Taira et al. 2004 (*13*)	Elbasha et al. 2007 (*9*)
Key assumptions in published models				
Target of HPV vaccine	HPV 16,18	High-risk HPV types	HPV 16,18	HPV 6,11,16,18
Efficacy of vaccine	90%	75%	90%	100%‡
Vaccine cost per series	$393	$300	$300 + $100 booster	$360
Base year of $US	2002	2001	2001	2005
Estimated cost per QALY of vaccination				
Published model estimate	$24,300	$12,700§	$14,600	$3,000
Simplified model estimate	$20,600	$8,700	$17,100	$5,300

*QALY, quality-adjusted life year; HPV, human papillomavirus.
†In all comparisons, the simplified model was modified (as necessary) so that the assumptions regarding the target of the HPV vaccine, vaccine efficacy and cost, vaccine duration of protection (except in the comparison to Taira and colleagues [*13*], as noted in the online Technical Appendix, available from www.cdc.gov/EID/content/14/2/244-Techapp.pdf), and the base year of US$ were consistent with the published models (online Technical Appendix). The simplified model estimate was based on the cohort model in the comparisons with the findings of Goldie et al. (*10*) and Sanders and Taira (*11*) and was based on the population model (assuming transmission effects) in the comparison with the estimates of Taira and colleagues (*13*) and Elbasha and colleagues (*9*).
‡Elbasha and colleagues (*9*) assumed 90% protection against infection with HPV and 100% protection against HPV-related disease.
§To enhance comparability, the published estimate from Sanders and Taira (*11*) was based on their sensitivity analyses when assuming lifetime duration of vaccination, not their base-case estimate of $22,800 when 10-y vaccine duration of protection was assumed.

vaccination and is not suitable for examining strategies such as vaccination of boys and men. In addition, although many of the parameter values and assumptions in our model can be modified with ease, changing the assumption of lifelong duration of protection or examining vaccination at older ages would require the incorporation of assumptions about the incidence and natural history of HPV to account for the probability of acquiring HPV (before vaccination or after vaccine immunity wanes) and the subsequent probability of adverse HPV-attributable health outcomes. However, we can address the issue of waning immunity by assigning a higher cost per vaccination series (as in the sensitivity analyses) to reflect the cost of a booster.

Another limitation of our approach is the uncertainty in the key parameter values, such as the cost and loss in quality of life associated with HPV-related health outcomes, the percentage of health outcomes attributable to each type of HPV targeted by the vaccine, and the incidence of CIN and genital warts. However, our results were fairly robust in response to changes in these key parameter values. For example, when simultaneously varying the costs of HPV-related health outcomes and the loss in QALYs associated with HPV-related health outcomes, we found that the estimated cost per QALY gained by vaccination ranged from $3,262 to $21,779.

Our adjustments for the effect of herd immunity were arbitrary; we simply assumed an additional effect of vaccination in the nonvaccinated population. However, our results did not vary substantially (in absolute terms) when the assumed effect of herd immunity was varied. For example, the estimated cost per QALY gained by quadrivalent vaccination (including herd immunity and excluding the benefits of preventing cancers other than cervical) was $5,336 in the base case and ranged from $3,423 to $7,596 when the adjustments for the effects of herd immunity (including the impact on genital warts in males) were varied. We also note that the benefits to nonvaccinated persons were assumed to occur only in nonvaccinated persons of similar ages to those vaccinated. This restriction may have understated the potential benefits of herd immunity.

Our analysis did not address all of the potential costs and benefits of vaccination. For example, the cost-effectiveness estimates would have been more favorable to vaccination if we had included the potential for cross-protection against high-risk HPV types besides 16 and 18 (*21*); the prevention of anal, vaginal, and vulvar cancer precursor lesions (as demonstrated in the supplemental analysis in the online Technical Appendix); the prevention of other cancers not included in this analysis (such as anal cancer and oropharyngeal cancers in male patients); and the prevention of other HPV-related health outcomes such as recurrent respiratory papillomatosis. Conversely, the cost-effectiveness estimates would have been less favorable to vaccination if we had included the potential for HPV type replacement (i.e., an increase in HPV types not protected against by vaccination), waning immunity, and the possible costs and loss in quality of life associated with adverse side effects of vaccination.

A key finding from this analysis was that the choice of discount rate and time horizon has a substantial influence on the estimated cost-effectiveness of vaccination. Because the costs of HPV vaccination begin to accrue immediately but the full benefits of vaccination are not realized for many years, the cost-effectiveness of vaccination becomes less favorable when higher discount rates are applied or when shorter time horizons are examined.

Another key finding was that the potential benefits of preventing anal, vaginal, vulvar, and oropharyngeal cancers offer nontrivial improvements in the estimated cost-effectiveness of HPV vaccination. The inclusion of these

additional benefits decreased the cost per QALY gained by vaccination by ≈$2,200 (or 21%) in the population model (without herd immunity), by ≈$1,400 (or 27%) in the population model (with herd immunity), and by ≈$2,200 (or 25%) in the cohort model. Future studies that develop better estimates of the cost and loss in quality of life associated with these cancers could more accurately estimate the effects of these additional benefits on the cost-effectiveness of HPV vaccination. Despite the limitations discussed above, our simplified model provides useful estimates of cost-effectiveness of HPV vaccination in the United States. Our results were consistent with previous studies based on more complex models. This consistency is reassuring because models of various degrees of complexity will be essential tools for policy makers in the development of optimal HPV vaccination strategies.

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Genetic Characterization of Feline Leukemia Virus from Florida Panthers

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From 2002 through 2005, an outbreak of feline leukemia virus (FeLV) occurred in Florida panthers (*Puma concolor coryi*). Clinical signs included lymphadenopathy, anemia, septicemia, and weight loss; 5 panthers died. Not associated with FeLV outcome were the genetic heritage of the panthers (pure Florida vs. Texas/Florida crosses) and co-infection with feline immunodeficiency virus. Genetic analysis of panther FeLV, designated FeLV-Pco, determined that the outbreak likely came from 1 cross-species transmission from a domestic cat. The FeLV-Pco virus was closely related to the domestic cat exogenous FeLV-A subgroup in lacking recombinant segments derived from endogenous FeLV. FeLV-Pco sequences were most similar to the well-characterized FeLV-945 strain, which is highly virulent and strongly pathogenic in domestic cats because of unique long terminal repeat and envelope sequences. These unique features may also account for the severity of the outbreak after cross-species transmission to the panther.

The Florida panther (*Puma concolor coryi*) is the only remaining puma (also called cougar or mountain lion) population east of the Mississippi River in North America. This population, which is confined to a small portion of southern Florida, was originally described as 1 of 30 subspecies of puma (*1*). By the 1970s, Florida panther numbers diminished to ≈30 because of hunting and habitat destruction. Since the early 1980s, the population has been studied extensively by monitoring a large proportion of adults by radio telemetry (*2*–*5*). In the early 1990s, concern over the fate of the population increased as signs of inbreeding and loss of genetic diversity were reported. These observations included low levels of genetic variation, high levels of sperm abnormalities, and increased incidence of heart defects relative to other puma populations and felids in general (*2*,*3*). In 1995, faced with the compounding effects of reduced genetic variation, probable depression of numbers from inbreeding, and evidence of compromised health, wildlife managers released 8 female Texas pumas into southern Florida to increase genetic variation and ameliorate the physiologic effects of inbreeding. Subsequently, increases were noted in the population of individuals of mixed genetic heritage, genetic variation, and population size; a decrease was noted in incidence of deleterious physiologic traits in crosses between the pure Florida panthers and the Texas females (*4*).

The Florida panther population, as well as other North and South American puma populations, has historically tested negative for exposure to or infection by feline leukemia viruses (FeLVs). A serosurvey of 38 free-ranging Florida panthers sampled during 1978–1991 reported complete absence of FeLV antigen (*3*). However, since early 2001, 23 panthers (>33% of the population) were found to be positive for FeLV antibodies, and at least 5 adult panthers were positive for FeLV antigen and subsequently died. In the 3 panthers available for necropsy, evidence was found of diseases compatible with FeLV infection (*5*). We describe the molecular genetic characterization of circulating FeLV strains isolated from the 2001–2005 outbreak and compare them with FeLV strains isolated from domestic cats.

FeLV is transmitted horizontally among domestic cats through body secretions (*6*) and was the first retrovirus shown to cause both neoplastic and degenerative disorders (*7*,*8*). Like other retroviruses, FeLV induces im-

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munosuppression in its host. Although the mechanism of immunopathogenesis is unclear, viral envelope proteins may be involved (*9*). FeLV envelope (*env*) and the long terminal repeat (LTR) sequences have been suggested as being involved in determination of disease sequelae, virus transactivation, and virus replication (*10–12*). There are 4 naturally occurring viral subgroups of exogenous FeLV (A, B, C, and T) that are distinguished genetically by sequence differences in the *env* gene and functionally by receptor interactions required for cell entry (*13*). FeLV-A is the predominant subgroup circulating in feral cats and is often only weakly pathogenic (*14*). FeLV-B, -C, and -T subgroups arise in vivo through recombination between exogenous FeLV strains and domestic cat endogenous FeLVs (*8,15*). The endogenous feline leukemia provirus sequences are present in the genome of the domestic cat and are transmitted vertically as integral components of the germline (*16*). Endogenous feline leukemia virus sequences by themselves do not produce infectious virus. However, the pathogenic subgroups, FeLV-B, -C, and –T, are generated by recombination in the *env* region between exogenous subgroup A virus and endogenous proviral sequences (*8*). FeLV-A, -B, -C, and -T are often associated, respectively, with thymic lymphoma of T-cell origin (*17*), tumor formation (*18*), aplastic anemia and bone marrow dysfunction (*17*), and lymphoid depletion and immunodeficiency disease (*13*). We used viral genome sequence and phylogenetic analyses to identify and characterize the virulent and pathogenic FeLV in Florida panthers and compare it with FeLV strains in the domestic cat.

Materials and Methods

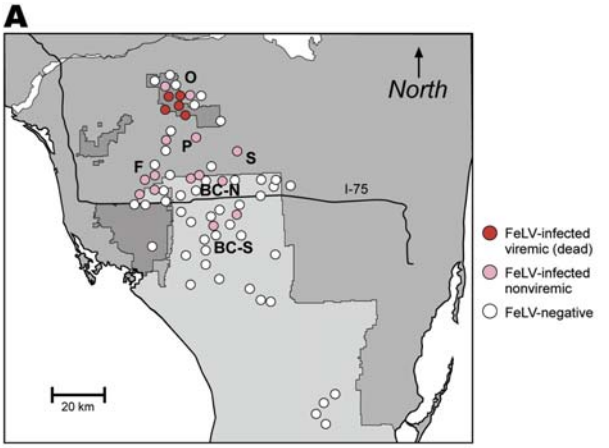
Sample Collection and Testing

Blood and tissue samples were collected from 61 free-ranging pumas captured during 1988–2004, mainly from south Florida. Samples were stored at –70°C and tested for FeLV antigen and antibody and for feline immunodeficiency virus (FIV) by Western blot as described (*5*) (Figure 1).

PCR Amplification of Proviral DNA

Genomic DNA was isolated from leukocytes, lymph nodes, spleen, intestines, or bone marrow of 61 panthers, including all that were positive for FeLV antigen and antibody. Proteinase K digestion was followed by standard extraction using the QIAGEN DNeasy tissue DNA extraction kit (#69504; QIAGEN, Valencia, CA, USA). Isolated DNA was visualized by electrophoresis on a 1% agarose gel and quantified by using a UV spectrophotometer (Bio-Rad, Hercules, CA, USA). PCR primers were designed from the conserved regions of *env* and LTR sequences of domestic cat FeLV (GenBank accession nos. M18247, M18248, M12500, AY374189, X00188, M14331, M23025, AY364318). PCR primers amplifying *env* (437 bp and 1,700 bp) and *env*/LTR (725 bp) are listed in Figure 2. The forward *env*/LTR primer (LTR4) was designed by using panther FeLV (FeLV-Pco) envelope sequence additionally.

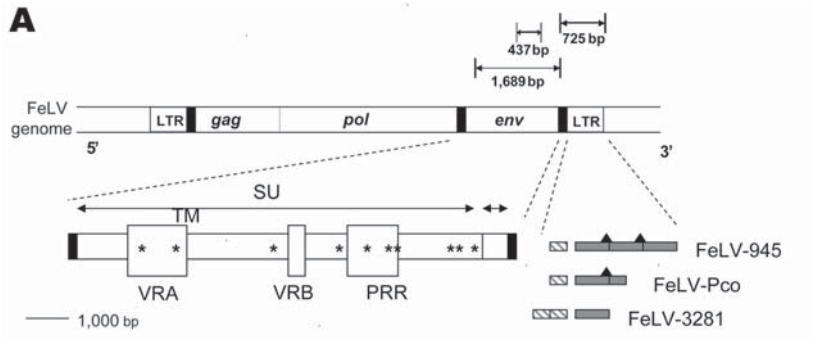
PCR was performed by using ≈50 ng of genomic DNA in a 50-μL reaction with 50 mmol/L KCl; 10 mmol/L



B

LDG ID	FP ID	Sex	1999	2000	2001 Jan–Jun	2001 Jun–Dec	2002 Jan–Jun	2002 Jun–Dec	2003	2004 Jan–Jun	2004 Jun–Dec	2005	GH	FIV	GEO
Pco-1058	FP-115	F						+,+,+	+,+,*				C	+	O
Pco-1022	FP-109	M					–,*,–		+,+,+				C	–	O
Pco-1087	FP-122	F								+§,+,–			C	–	O
Pco-1088	FP-123	M								+,+,–	+,+,*		H	+	O
Pco-1098	FP-132	M								–,*,–	+,+,*		H	–	O
Pco-722	FP-167	F	*,*,–				–,–,+						C	–	P
Pco-908	FP-78	F	*,–,–			–,–,+							C	+	F
Pco-962	FP-82	F		*,*,–				–,–,+					C	–	O
Pco-972	FP-96	M			–,+,–	–,+,+	*,+,*						C	+	O
Pco-990	FP-99	F			*,–,+	*,*,+							C	+	F
Pco-1016	UCFP43	M				–,–,+							H	+	P
Pco-994	FP-108	M				–,–,+							H	+	BC–S
Pco-1060	FP-118	F							–,+,–				H	–	S
Pco-1000	FP-104	M			–,–,+			*,*,–				*,*,–	H	+	BC–N
Pco-991	FP-100	M			–,+,+					–,+,*			C	–	BC–N
Pco-971	FP-107	F				–,–,+					*,*,–		C	+	F
Pco-984	FP-110	F					*,–,+	–,+,–					C	–	O
Pco-1064	FP-119	M							–,+,–		–,–,–		C	+	BC–S
Pco-901	FP-69	F										–,–,+	C	+	BC–N

Figure 1. A) Prevalence and distribution of 19 Florida panthers, sampled 1999–2005, showing evidence of feline leukemia virus (FeLV) exposure. All antigen-positive panthers (red) are clustered in the Okaloacoochee Slough State Forest (O). PCR-positive and/or antibody-positive (pink) pumas were found there also, as well as in the surrounding areas including Florida Panther National Wildlife Refuge (F), private lands (P), Big Cypress Seminole Indian Reservation (S), and Big Cypress North and South (BC-N, BC-S, respectively). All but 2 infected panthers were found north of Interstate 75. B) Information on affected panthers. Gray shading indicates timeline for monitoring of individual panthers until death. Symbols within gray boxes indicate presence (+), absence (–), or no data (*) for FeLV antigen in serum, FeLV sequence recovered by PCR, or presence of antibodies against FeLV in serum, respectively. FP-122 was antigen negative when tested 1 month previously (§). LGD ID, Laboratory of Genomic Diversity identification number; FP ID, Florida panther identification number; GH, genetic heritage; FIV, feline immunodeficiency virus; GEO, geographic locale; *, missing data; gray shading, lifespan of panthers; C, canonical (pure) Florida panther; H, Texas hybrid.



B

Forward primer (3′→5′)	Reverse primer (3′→5′)	gene	size	Name
AACARAAGTAAAGACTGTTGG	GCYTGGTGGGTCTTAGGAA	*env*	437 bp	PfeF6/PfeR6
TCTATGTTAGGAACCTTAACCGATG	TTAAGGCTTGTACCACAGATATTCTG	*env*	1,689 bp	PEF2/R14
AAGTCCCCCTGGCTTACAAC	GGAGACCTAGTTCAGGGGTCTT	*env*/LTR	725 bp	LTR4F/2R

Figure 2. A) Diagram of the feline leukemia virus (FeLV) genome showing the PCR products obtained from FeLV-Pco *env* and long terminal repeat (LTR) genes. Envelope gene surface (SU) and transmembrane (TM) subunits, variable regions A and B (VRA and VRB) and the proline-rich region (PRR), 3' LTR enhancer element(s) (hatched rectangle), signature 21-bp repeat(s) (gray shading), and putative c-Myb binding sites (black triangles) (*12*) are depicted for FeLV-945, FeLV-Pco, and FeLV-3281A . Unique signature amino acid residues found only in FeLV-945 and FeLV-Pco are marked by asterisks (see Figure 5). B) Primer pair PfeF6/PfeR6 was designed to detect all FeLV subgroups.

Tris-HCl (pH 8.3); 1.mmol/L $MgCl_2$, 0.25 mmol/L each of dATP, dCTP, dGTP, and dTTP; 2 mmol/L of each primer; and 2.5 U of Taq Gold polymerase (Applied Biosystems, Foster City, CA, USA). PCR was run on a GeneAmp PCR system 9700 thermocycler (Applied Biosytems) under the following conditions: 9 min 45 s at 95°C; then a touchdown of annealing temperatures to reduce nonspecific amplication, always starting with 20 s at 94°C; then 30 s at 60°C (3 cycles), 58°C (5 cycles), 56°C (5 cycles), 54°C (5 cycles), 52°C (5 cycles), or 50°C (22 cycles), and then 30 s (437-bp *env)*, 1 min (LTR) or 2 min 20 s (1,698-bp *env*) at 72°C for extension; and a final extension at 72°C for 7 min. PCR products were examined after electrophoresis on a 1% agarose gel. Primers and unincorporated deoxynucleotide triphosphates were removed by using Microcon YM (Millipore, Billerica, MA, USA) technology or exonuclease I and shrimp alkaline phosphatase (Amersham, Piscataway, NJ, USA) (Figure 2). Representative PCR products from independent amplifications were cloned and sequenced. For the *env* and LTR sequences, products were cloned from 4 PCR products each (Figure 2). Cloning was performed with a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA was isolated from 6 to 16 clones from each reaction product by using a QIAGEN Miniprep Kit. Sequences were obtained from clones by using internal primers in standard ABI BigDye terminator (Applied Biosystems) reactions. Anticontamination measures were taken at all steps of PCR amplification and after PCR processing. Pre-PCR setup was performed in a laminar flow hood, DNA was added in a free-standing containment hood in a separate room, and all post-PCR manipulations were performed under a fume hood in a third room. All surfaces were washed with a 10% bleach solution, and each hood was exposed to UV light for 30 min before and after use. PCR tubes with individual lids, rather than 96-well plates, were used and kept closed except when reagents and DNA were being added or aliquots were extracted for use. DNA tubes were opened only under their designated hoods; to avoid cross-contamination, tubes were never open simultaneously. Water and a sample from an FeLV-negative puma were run with every reaction as negative controls. Positive controls of known sequence were also run for each reaction: 1 from a domestic cat, 1 from a known seropositive Florida panther (FP-115 or FP-122), or both.

Phylogenetic Analysis

Sequences from *env* and LTR were analyzed separately. For analysis relative to known domestic cat FeLV sequences, we included FeLV-945, FeLVA-3281, FeLVA-61E, FeLVA-Glasgow-1, FeLVC-Sarma, FeLVB-Rickard, SM-FeSV, enFeLV-AGTT (accession nos. AY662447, M18248, M18247, M12500, M14331, X00188, M23025, AY364318, respectively) (*env*) and FCA-945, FCA-934, FeLVA-3281, and FeLVA-Glasgow-1 (accession nos. AY374189, AY374184, M18248, and M12500, respectively) (LTR). Nucleotide sequences were compiled and aligned for subsequent phylogenetic analysis by ClustalX (*19*) and verified visually (*20*). MODELTEST (*21*) was used for *env* and LTR analysis to estimate the optimal model of sequence evolution; these settings were incorporated into subsequent analyses. Minimum-evolution trees were constructed from models of substitution specified by MODELTEST; starting trees were obtained by the neighbor-joining method, followed by application of a tree-bisection-reconnection branch-swapping algorithm during a heuristic search for the optimal tree. Maximum-parsimony analysis used a heuristic search of starting trees obtained by stepwise addition and followed by tree-bisection-reconnection. Maximum likelihood parameters specified by MODELTEST selected the general time-reversible model of substitution; they included empirical base frequencies and estimated rate matrix and corrected for among-site rate variation (γ distribution). A bootstrap analysis that used

1,000 iterations was performed with each method. Amino acid residue alignments were generated by using MacClade 3.05 (*20*) and ClustalX. Sequences were inspected for homoplasies. Nucleotide sequences were translated to protein, and genetic distances were calculated in MEGA 3.0 (*22*) by using the Tajima-Nei (nucleotide) and Dayhoff (amino acid) algorithms. The sequences of FeLV-Pco *env* and LTR were deposited in GenBank under accession nos. EU189489–EU189498.

Results

FeLV Serosurvey and PCR Amplification

The first sign of an emerging outbreak of FeLV in the free-ranging Florida panther population was the 2001 detection of FeLV antibodies, FeLV proviral PCR, or both, in 8 pumas from the Florida Panther National Wildlife Refuge, private lands, or the northern range of Big Cypress Swamp (Figure 1). Antigen-positive results and documented death compatible with FeLV infection first occurred in FP-115 in 2002 near the Okaloacoochee Slough State Forest (*5*). With the exception of FP-108 and FP-119, found in the central region of Big Cypress National Park, all 19 other FeLV-exposed panthers were found north of Interstate 75 (Figure 1) (*5*). During the next 2 years, 4 additional antigen-positive panthers died; FeLV-related disease was suspected for 2 (FP-123 and FP-132) and confirmed for 2 (FP-109 and FP-122) (*5*). Additionally, 8 panthers (FP-67, FP-78, FP-82, FP-96, FP-99, UCFP43, FP-108, FP-118) that were antigen negative but seropositive or PCR positive for FeLV died during the outbreak, but their deaths were not attributed to FeLV (*5*).

Retrospective screening of 6 panthers (FP-67, FP-78, FP-82, FP-109, FP-122, FP-132) for antibody or antigen or by PCR demonstrated that they had not had FeLV infection before this outbreak. FP-96 in the Florida Panther National Wildlife Refuge was one of the first to have documented FeLV exposure; this panther displayed a latent infection, being PCR positive in 2001 and in 2002. Three panthers (FP-104, FP-107, FP-119) likely cleared the virus; after initial positive test results, they were seronegative on follow-up testing. Positive FIV antibody results by Western blot were found for 11 of the 19 FeLV-exposed and 2 of the 5 clinically affected panthers (Figure 1). An analysis of 21 microsatellites (short tandem repeats) showed that 6 of the 19 FeLV-exposed and 2 of the 5 antigen-positive panthers were crosses with some Texas heritage and that the rest were pure Florida panthers (W.E. Johnson et al., unpub. data).

Phylogenetic Analysis

An alignment of FeLV-Pco, FeLV-A, FeLV-B, and endogenous *env* nucleotide sequence (Figure 3) established the concordance of FeLV-Pco with subgroup A and found a lack of recombination of FeLV-Pco with endogenous FeLV-Pco sequence. The absence of endogenous sequences was not unexpected because pumas and other cats outside of the genus *Felis* do not carry endogenous FeLV sequences (*23*,*24*). The FeLV-Pco was classified as subgroup A on the basis of this lack of evidence for recombination with endogenous FeLV across 1,794 bp of FeLV-Pco *env* sequence (Figure 3) and on the basis of in vitro receptor utilization studies (*5*). The aligned sequences of the LTRs and the *env* region were analyzed as separate datasets. For both datasets, phylogenetic analyses identified the FeLV-Pco sequences as monophyletic (Figure 4). Each had strong bootstrap support for a clade containing all FeLV-Pco but none of the previously sequenced domestic cat FeLVs (Figure 4). This pattern was consistent with a recent and focal introduction of the virus. Furthermore, the 376-bp nucleotide *env* sequence obtained from the earliest cases of FeLV exposure (Pco-972 and Pco-991, found respectively in the Florida Panther National Wildlife Reserve and northern Big Cypress National Preserve), were identical in sequence to the later FeLV cases found in the Okaloacoochee Slough State Forest (Figure 2; online Appendix Figure 1, available from www.cdc.gov/EID/content/14/2/252-appG1.htm). On the basis of >50 cloned envelope sequences (Figure 4; Table), the FeLV-Pco viruses associated with this outbreak were highly conserved. The mean percentage nucleotide and amino acid sequence differences of the FeLV *env* gene among FeLV-Pco sequences were 0.4% (nucleotide) and 0.1% (amino acid). Of published FeLV sequences available in GenBank, the closest strain was the domestic cat

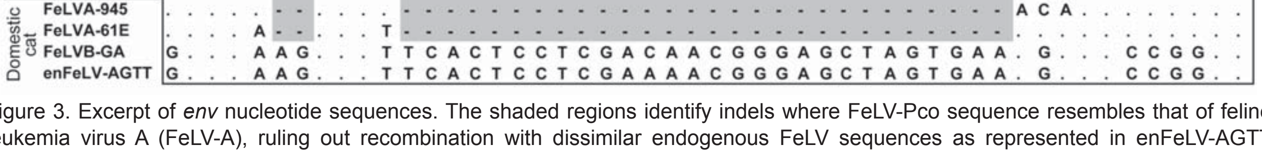


Figure 3. Excerpt of *env* nucleotide sequences. The shaded regions identify indels where FeLV-Pco sequence resembles that of feline leukemia virus A (FeLV-A), ruling out recombination with dissimilar endogenous FeLV sequences as represented in enFeLV-AGTT (bottom). Puma sequences, with year of sampling (for example FeLV-Pco-1058-03 was sampled in 2003); domestic cat subgroup A (FeLVA-945 and FeLVA-61E), recombinant (FeLVB-GA), and endogenous (enFeLV-AGTT) sequences are also shown. Matches to the reference sequence (Pco-1058-02) are indicated by a dot. Gaps are indicated by a dash. Expanded figure is available online from www.cdc.gov/EID/content/14/2/252-G3.htm

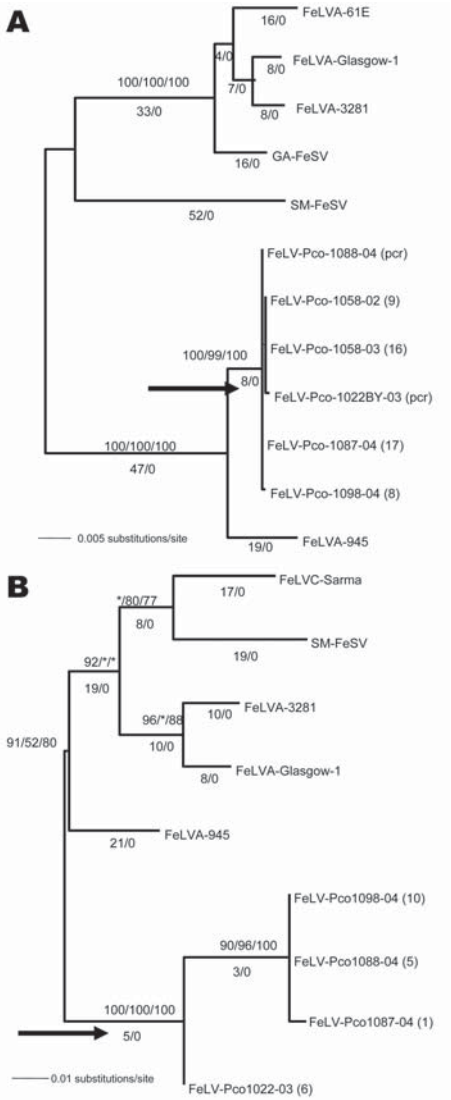


Figure 4. Phylogenetic trees of panther feline leukemia virus (FeLV-Pco) and domestic cat FeLV nucleotide sequences. A) Midpoint rooted maximum-likelihood phylogram based on 1,698 bp of *env* sequences. B) Midpoint rooted maximum-likelihood phylogram based on 463 bp of 3' long terminal repeat (LTR) sequences. Consensus FeLV-Pco sequences of clones generated from 5 *env* and 4 LTR panthers and reference domestic cat sequences are shown. The number of FeLV-Pco–cloned PCR products used in each consensus sequence is indicated in parentheses. The arrow indicates the monophyletic clade of all FeLV-Pco sequences. A similar topology, including the monophyletic clade, was obtained by using the different FeLV-Pco clone sequences rather than a consensus. The year of panther sampling is indicated as a suffix, e.g., Pco-1088-04 was sampled in 2004. Where maximum-likelihood tree was congruent with maximum parsimony tree, branch lengths are indicated below branches. Number of homoplasies is indicated after the branch length. Bootstrap values are shown (maximum parsimony/minimum evolution/maximum likelihood). Asterisk (*) indicates bootstrap value <70. The score (–ln likelihood) of the best maximum-likelihood tree was *env* 3615.01706, LTRs 1836.05922 (best tree found by maximum parsimony: *env* length = 221, consistency index [CI] = 0.941, retention index [RI] = 0.963; LTR length = 132, CI = 0.871, RI = 0.787).

virus FeLV-945, according to LTR and *env* sequence comparisons (Figure 4); calculated differences were only 1.5% (nucleotide) and 3.5% (amino acid) between FeLV-Pco and FeLV-945 *env* sequences (Table).

Because FeLV-945 is well characterized and highly virulent in the domestic cat (*11*,*25*), sequence elements associated with disease determination (*env*) and transcription enhancement (LTR) in FeLV-945 were examined in FeLV-Pco. In the envelope protein, 10 signature amino acid residues (found within the surface glycoprotein) that were shared between FeLV-Pco and FeLV-945 were distinctive from other strains of FeLV (Figure 5). Of these synapomorphic sites, 2 were in variable region A, which in FeLV-945 defines the specificity required for viral binding to receptors (*25*). Three of the sites were within the proline-rich region, which in FeLV-945 encodes for conformational changes required for FeLV cell entry (*25*). The FeLV-Pco LTR sequences had 1 copy of a 40-bp enhancer element that has been characterized in FeLV-945 (online Appendix Figure 2, available from www.cdc.gov/EID/content/14/2/252-appG2.htm) (*12*). Finally, the exogenous domestic cat FeLV-945 isolate, which FeLV-Pco strains resemble (Figures 4, 5), displays unusual repeat junctions where the transcription factor c-Myb is known to bind in FeLV-945, possibly accelerating the rate of transcription of the virus (Figure 2, online Appendix Figure 2) (*12*). FeLV-Pco also contains 1 copy of this repeat junction (Figure 2, panel A; online Appendix Figure 2), which supports the conclusion that FeLV-Pco is derived from a strain closely related to and perhaps from the pathogenic FeLV-945 domestic cat strain. FeLV-945 is unusual in that its severe pathogenicity does not involve recombination with endogenous FeLV in domestic cats. That FeLV-Pco pathogenesis in pumas is due to a virus similar to FeLV-945 that was not derived from endogenous recombination is consistent with the complete lack of endogenous FeLV sequences in the puma genome.

Discussion

We genetically characterized the FeLV that emerged in the previously naive free-ranging Florida panther population. According to the retrospective longitudinal antibody and antigen results and the virus' geographic distribution, the virus was likely introduced into the Florida panther population in 2001 (Figure 1) (*3*). From the earliest detected panthers with FeLV (2001) to the most recent (2005), the FeLV-Pco *env* sequences were nearly identical, which indicates that the source of infection was likely a single domestic cat. FP-96, resident in the Florida Panther National Wildlife Reserve area in January of 2001, was the first panther with exposure detectable by PCR. The virus then spread north and east through the population, affecting individual panthers in Big Cypress (FP-100, FP-119), Seminole Indian Reservation (FP-118), and Okaloacooch-

Table. Mean percent amino acid and nucleotide *env* sequence differences of feline leukemia virus subgroups, FeLV-945, and FeLV-Pco strains*

Strain	FeLV A†	FeLV B‡	FeLV C§	FeLV-945¶	FeLV Pco#
FeLV A	**1.8**, 3.8	10.3	6.6	6.4	6.1
FeLV B	**19.1**	NA	13.2	14	13.3
FeLV C	**16.3**	**28.7**	**14.2**, 6.2	7.3	7.4
FeLV 945	**15.2**	**30.1**	**16.4**	NA	1.5
FeLV Pco	**14.3**	**28.2**	**16.7**	**3.5**	**0.4**, 0.1

*FeLV, feline leukemia virus. Shaded gray boxes contain mean percent sequence differences within strain. **Boldface** indicates mean percent amino acid *env* sequence differences, 1,689 bp (see Figure 2). Nucleotide differences are above diagonal.
†M18247, M18248, M12500, AF052723.
‡X00188.
§M14331, M23025
¶AY374189.
#Pco-1058-02 (9 cloned sequences), Pco-1058-03 (16 cloned sequences), Pco-1087-04 (17 cloned sequences), Pco-1098-04 (8 cloned sequences).

ee Slough (FP-109, FP-108, FP-115, FP-122, FP-123, FP-132) (Figure 1; online Technical Appendix, available from www.cdc.gov/EID/content/14/2/252-Techapp.htm). Texas genetic heritage did not protect infected pumas from disease associated with FeLV; pure Florida panthers and pumas died after having symptoms compatible with FeLV (Figure 1).

Among characterized strains of FeLV, domestic cat FeLV-945 was closest in sequence to FeLV-Pco in the panthers. FeLV-945 in domestic cats was originally isolated as the predominant FeLV species from a geographic cohort of 21 infected domestic cats and is known to cause non–T-cell diseases characterized by degenerative and proliferative changes of myeloid and erythroid origin (*26*). Although FeLV-945 is included among FeLV subgroup A isolates on the basis of cell receptor utilization, its distinctive envelope and LTR sequence signatures differ from those of other FeLV-A strains (*25*). At the amino-terminal of the envelope sequence, the surface glycoprotein, also known as gp70, encodes the receptor-binding domain, within which are 2 variable regions, A and B. These define the specificity required for binding. Further downstream, a proline-rich region encodes for the conformational changes required for viral entry (*25*).

The 10 envelope amino acid residues synapomorphic in FeLV-Pco and FeLV-945 included 2 in variable region A and 3 in the proline-rich region (Figures 2, 5). In FeLV-945 LTR, three 21-bp repeats form 2 junctions: 1 junction is formed by the first repeat and the adjacent second repeat; the other is formed by the second and third repeats. Each junction includes a c-Myb binding site that increases the rate of viral replication through the recruitment of transcriptional coactivator binding protein (cAMP response element) (*11*). FeLV-Pco LTR sequences had 1 copy of the repeat junction (Figure 2, online Appendix Figure 2) (*12*). Upstream, LTR transcriptional enhancer elements repeated in tandem have been associated with thymic lymphomas and are found only in 1 copy in non–T-cell disease (*26*). Like FeLV-945, FeLV-Pco lacks this duplication (Figure 2, online Appendix Figure 2).

In the panthers, clinical and pathologic findings of FeLV-Pco in this outbreak consisted of FeLV-related diseases of non–T-cell origin. These findings are consistent with the pathologic changes associated with FeLV-945 in the domestic cat. Necropsy findings of FP-115 documented interstitial pneumonia, septicemia, and suppurative lymphadenopathy. Examination of FP-109 1 month before it died found lymphadenopathy, anemia, lymphopenia, and lymphoid hyperplasia. FP-122 had similar findings 1 month before it died, including lymphadenopathy, muscle wasting, and hypercellular bone marrow with >90% hematopoietic cells. FP-132 necropsy findings included severe pallor (indicative of anemia), bronchointerstitial pneumonia, abscesses, lymphadenopathy, and hypercellular bone marrow with >90% hematopoietic cells (*5*). FeLV-Pco is therefore similar to the unique and virulent domestic cat strain FeLV-945 of FeLV subgroup A, in *env* and *LTR* sequence and in non–T-cell disease outcome. In the domestic cat, FeLV-

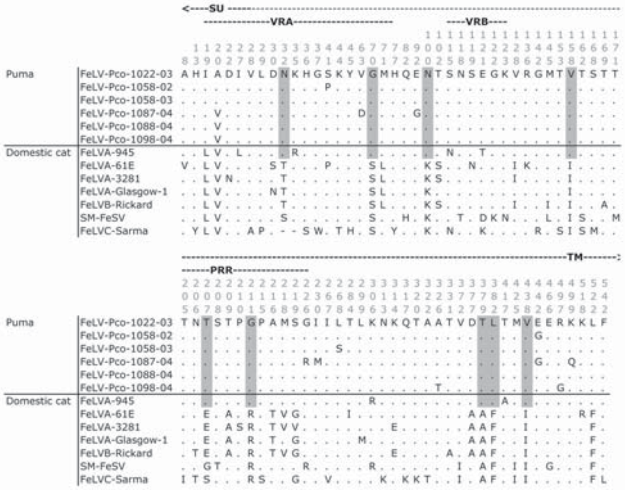


Figure 5. Variable sites in the amino acid alignment of panther feline leukemia virus (FeLV-Pco) and domestic cat FeLV *env* sequences (1,689 bp). Surface glycoprotein (SU), transmembrane (TM), variable region A and B (VRA and VRB), and proline-rich region (PRR) locations are indicated. Horizontal line separates sequences of puma (above) and domestic cat (below). The 10 amino acid residues in this region unique to FeLV-945 and FeLV-Pco sequences are highlighted. Matches to the reference sequence are indicated by dots; gaps are indicted by dashes.

945 causes multicentric lymphoma, myeloproliferative disorder, and anemia and has never been associated with thymic lymphoma (*26*). These findings shared between FeLV-945 and FeLV-Pco implicate the 10 identified amino acid synapomorphies (Figure 5) as plausible determinants of disease. Further study of these *env* regions from T-cell and non–T-cell disease manifestations of FeLV occurring in comparative felid species is warranted and may elucidate the key sequence determinants of disease outcome in FeLV.

The role of FIV-related immune suppression, if any, in this outbreak is uncertain. Although recent studies of T-lymphocyte profiles in FIV-infected wild lions and pumas suggest that CD4 depletion occurs (*27*), our survey found that co-infection with FIV was present in 2 but absent in 3 FeLV-associated deaths. FIV-positive panthers could have served as a reservoir for the spread of FeLV through the population because the earliest detected FeLV-exposed panthers (FP-96 and FP-99) were FIV positive. Furthermore, the first panther (FP-115) detected with FeLV-compatible disease in the Okaloacoochee Slough State Forest region was positive for FIV and FeLV for at least 6 months.

An FIV serosurvey suggested an overall increase in the prevalence of FIV in Florida panthers in recent years. During 1999–2000, 3 (15%) of 20 panthers tested had FIV-positive results by Western blot. In contrast, 13 (76%) of 17 panthers tested during 2004–2005 in the FeLV-endemic Okaloacoochee Slough State Forest region (Figure 1) were FIV positive (*5*). These results could support a role for FIV-mediated immune depletion in FeLV pathogenesis. In domestic cats, FIV and FeLV co-infections have resulted in conflicting interpretations (*28*–*32*). In contrast to FIV, which is found in many species of wild felids (*33*), FeLV in nondomestic felids has been reported only a few times, in captive cats, with documented or suspected exposure to infected domestic cats (*5*). Serologic survey of free-ranging populations found an absence of FeLV in pumas in California (*34*), among felids in Botswana (*35*), and among 38 free-ranging Florida panthers sampled during 1978–1991 (*3*). However, Jessup et al. (*36*) document a case of FeLV in a young adult male free-ranging puma captured from a college campus in Sacramento, California. Necropsy of this cougar found generalized lymphadenopathy and lymphoproliferative disease. These necropsy results are consistent with and similar to the clinical findings of the FeLV-positive panthers reported here.

The outbreak of FeLV in the previously naive population of endangered Florida panthers raised questions about management of free-ranging pumas. In response, the Florida Department of Fisheries and Wildlife began a widespread vaccination program of Florida panthers; no additional FeLV cases have since been detected among them (*5*).

This emerging disease outbreak was characterized by 2 factors. First, because of its unique heritage and popularity, the Florida panther has been the most intensively monitored wild felid in North America. Second, the extensive veterinary surveillance of the domestic cat has provided powerful models for studying infectious diseases relevant to understanding human health and disease, including retroviruses such as FeLV (*37*). Although future cross-species transmission events among wild and domestic carnivore populations may be unavoidable, our understanding of pathogen and host genetic determinants may also be greatly enhanced by the recent release of the genome sequence of the domestic cat (*38*). Combining progress in biomedical genomics with intensive studies of wild species can provide insights into emerging pathogens that affect wild, domestic, and human hosts.

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We dedicate this article to Murry Gardner, who originally uncovered the FeLV-945 isolates in California and whose accomplishments and career as a retrovirology pioneer have inspired generations of veterinary scientists.

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Dr Brown is a veterinarian and a PhD candidate research fellow in the Laboratory of Genomic Diversity at the National Cancer Institute in Frederick, Maryland. Her primary research interests include molecular genetic determinants of viral pathogenesis in the cat model.

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Diagnosis of Cystic Echinococcosis, Central Peruvian Highlands

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We evaluated prevalence of cystic echinococcosis (CE) in a central Peruvian Highland district by using 4 diagnostic methods: ultrasonography for 949 persons, radiography for 829, and 2 serologic tests for 929 (2 immunoblot formats using bovine hydatid cyst fluid [IBCF] and recombinant EpC1 glutathione S-transferase [rEpC1-GST] antigens). For the IBCF and rEpC1-GST testing, prevalence of liver and pulmonary CE was 4.7% and 1.1% and seropositivity was 8.9% and 19.7%, respectively. Frequency of seropositive results for IBCF and rEpC1-GST testing was 35.7% and 16.7% (all hepatic cysts), 47.1% and 29.4% (hepatic calcifications excluded), and 22.2% and 33.3% (lung cysts), respectively. Weak immune response against lung cysts, calcified cysts, small cysts, and cysts in sites other than lung and liver might explain the poor performance of the serodiagnostic tests. We confirm that CE is highly endemic to Peru and emphasize the limited performance of available serologic assays in the field.

Cystic echinococcosis (CE), caused by the larval stage of *Echinococcus granulosus*, is recognized as a public health problem (*1*). Cysts develop in internal organs of intermediate hosts (herbivores and humans). The disease represents a challenge of increasing concern in countries where control programs have been reduced or have not yet been implemented (*2*,*3*). Globally, the annual loss due to human hydatidosis (treatment and lost income) has been estimated at ≈US $200 million (*4*).

CE is endemic to ≥100 countries in Latin America, Asia, and Africa (*1*,*5*) and is considered an emerging disease in other areas. In the former Soviet Union and Eastern Europe, the number of cases has dramatically increased in recent years (*6*–*8*). The annual incidence of CE hospital cases has reached >8/100,000 persons in some European countries, and 42/100,000 in Xinjiang, People's Republic of China (*5*). The highest incidence of surgical cases (198/100,000) has been reported in Kenya (*1*). A few areas (Iceland, Ireland, and Greenland) are believed to be free of autochthonous transmission. The United States has reported a few cases in livestock; most CE infections in persons are imported. This is also true for regions of western and central Europe (*4*) with the exception of countries such as Spain, where the parasite is prevalent and remains a major public health problem (*9*,*10*).

Studies in Peru have shown high prevalence of CE in humans, particularly in the central and southern highlands (*11*,*12*). During 1997–1999, prevalence in the central Andes was 5.7%–9.3% according to ultrasonography, radiography, or both and up to 18.2% according to immunoblot testing (*11*,*12*). Portable ultrasonography has facilitated the study and more accurate reporting of CE prevalence in endemic regions (*13*,*14*), following the standardized World Health Organization classification (*15*).

Among available serologic tests, the immunoblot (IB) assay that uses bovine hydatid cyst fluid (IBCF) has been successfully used in CE-endemic areas of Peru (*12*,*14*). This IBCF has a sensitivity of 80% for hepatic cysts and 56% for pulmonary cysts (*16*). Another immunoblot test, which uses a purified recombinant EpC1 glutathione S-transferase antigen (rEpC1-GST), has a sensitivity of 92.2% and a specificity of 95.6% (*17*). An immunoreactive clone (*EpC1),* encoding EpC1 was identified by immunoscreening a cDNA library constructed with RNA extracted from protoscolices from sheep hydatid cysts. Immunoglobulin (Ig) G was the dominant antibody isotype generated against rEpC1-GST (*17*). To this point, no field testing of the EpC1

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had been undertaken. Measuring the real extent of CE in South America as well as evaluating the native, recombinant, and peptide antigens for diagnosis of CE in humans have been recommended (*18*). During July and August 2004, we used 4 diagnostic methods—ultrasonography, chest radiography, and 2 serologic assays—to evaluate the prevalence of CE in humans in an unexplored CE-endemic area of the central Peruvian Highlands where control measures have been attempted incompletely.

Materials and Methods

Study Site

We selected 9 rural communities located 5–50 km from Yanahuanca district, which is located in the Pasco department (central Peruvian Andes) at 3,249–4,314 m above sea level (Figure 1). The terrain is mountainous and roads are unpaved. Houses are made of adobe, and drinking water is obtained from streams or rivers. Primary healthcare is provided by health centers; specialized care is available from the closest hospital in Pasco (40–60 km).

Sheep (n = 99,175) are the dominant livestock, but cattle (n = 5,451), swine (n = 2,784), alpacas (n = 2104), llamas (n = 5,679), and guinea pigs (n = 8,870) (*19*) are raised for human consumption. With the exception of guinea pigs, animals are kept in fields distant from the villages. Dogs are routinely used as shepherds, but some are kept as pets.

Study Design

After coordination with local authorities, a census was taken of persons in each village; to maintain confidentiality, persons were assigned a code. A cross-sectional study was performed by using ultrasonography of the abdomen, radiography of the chest, and 2 immunoblot assays with different antigens (crude IBCF [*16*] and a recombinant antigen rEpC1-GST [*17*]). Persons ≥5 years of age were invited to participate. All examinations were conducted at community health centers; 3–4 mL of blood was taken from all persons who volunteered to participate in the study. Women of childbearing age were asked to have a urine pregnancy test, and those who were pregnant were excluded from radiographic examination. The ethics review boards of the Universidad Peruana Cayetano Heredia and the Bloomberg School of Public Health of Johns Hopkins University approved the study and written consent forms.

Radiography

Posterior-anterior portable radiographs were taken by using a Polyskop machine (Siemens, Orlando, FL, USA). A radiologist, who was not provided serologic and ultrasonography results, read the films and classified the findings by using the Beggs criteria for lung CE (*20*). Unruptured cysts were defined as centrally located, closed, well-defined, and round lesions.

Ultrasonography

Ultrasonography was performed with a portable 3.5-MHz ultrasonograph (model Shimasonic SLD-32, Shimatzu, Kyoto, Japan). Cysts were diagnosed by using the international classification of ultrasound images for CE, which classifies cysts in cystic lesions, and from CE type 1 to CE type 5 according to their development grade or degeneration (*15*).

IBCF Immunoblot

Bovine hydatid cysts were obtained from abattoirs, and fluid was aspirated and placed in a beaker at 4°C to which 0.5 M phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA) was added (1:100 dilution). After centrifugation at 3,000 *g* for 10 min, the supernatant was lyophilized and stored at –20°C until use. The antigen was diluted with 0.1% sodium dodecyl sulfate (SDS), 0.025 (w/v) bromophenol blue, 0.0025 M Tris-HCl (pH 8.0), and the dilution was completed with 6% glycerol to give a final concentration of 0.2 μg/μL. The antigen was resolved by polyacrylamide gel electrophoresis as described elsewhere (*16*,*21*). The separated proteins were electrotransferred to nitrocellulose membrane, cut into strips, and immersed in a dilution of 1:25 (serum:phosphate-buffered saline with 0.3% Tween 20). Bound human antibodies were detected by incubating each strip in horseradish peroxidase–conjugated goat antibody to human IgG at a dilution of 1:1,000. Antibodies bound to diagnostic bands of 8, 16, and 21 kDa were seen after addition of 3,3′-diaminobenzidine. A positive result was defined as the presence of any diagnostic band (*16*).

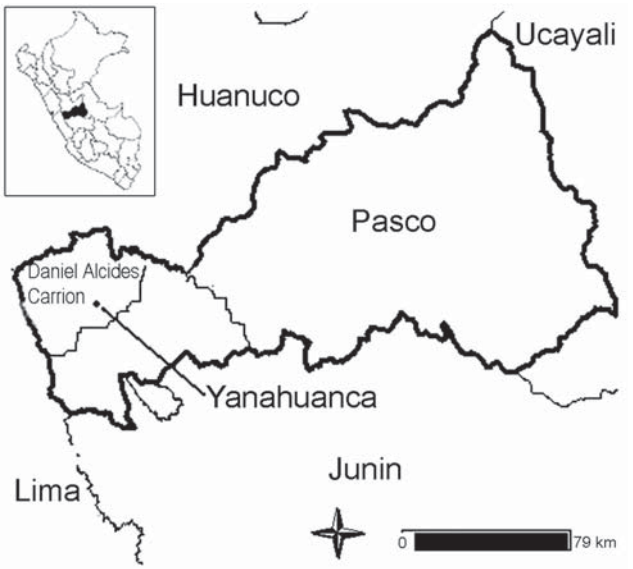

Figure 1. Map of the Central Peruvian Highlands.

rEpC1-GST Immunoblot

rEpC1-GST was expressed in *Escherichia coli* (*21*). The rEpC1-GST fusion protein was subjected to electrophoresis on 12% (w/v) SDS-polyacrylamide gels under reducing conditions and then transferred onto nitrocellulose membrane; the membrane was then cut into strips containing ≈0.3 μg of rEpC1-GST protein, as described (*17*). After being blotted with 5% (w/v) skim milk, the strips were incubated with human serum samples (diluted 1:100) for 1 h at 37°C and washed 3× with phosphate-buffered saline Tween before being incubated with goat anti-human whole immunoglobulin IgG conjugate (Sigma). After development in 4-chloro-1-naphanol substrate solution for 15 min at room temperature, the strips were examined. A positive serum sample showed a band of ≈41 kDa (*17*).

Data Analysis

The prevalence of CE was determined for the 2 imaging techniques, and the proportion of seropositive persons was estimated according to serologic test results. The test prevalence was calculated for all communities, and the difference was assessed by a 2-sample test of proportions. The χ^2 test was used to evaluate the association of sex with positive results for all 4 tests. The frequency of seropositivity (by IBCF or rEpC1-GST) for persons who had CE-positive ultrasonographic or radiographic images was calculated to evaluate the performance of the immunoblot tests. This frequency was, moreover, assessed with and without hepatic calcified cysts, and for liver cysts ≤20 mm (24 persons) or >20 mm (18 persons) in diameter. The κ test was used to establish the agreement between the 2 serologic assays and with either ultrasonography or radiography. All statistical analyses were computed by using Stata 8.0 (Stata Corporation, College Station, TX, USA) with a significance level of <0.05.

Results

Of the 1,973 persons registered during the census, 137 (7%) were <5 years of age, which left 1,836 potential study participants. Of these, 949 persons (51.7%) were examined. All 949 were evaluated with ultrasonography, 829 had chest radiographs taken, and 929 contributed blood samples. The proportion of females was higher among participants (60.3%) than nonparticipants (50%) (p<0.05). Of those with ultrasonographic results, 39 had no serologic results because they refused to have their blood collected. In addition, 125 persons had serologic results but refused to have chest radiographs taken. The ages of participants and community members were similar (mean age 28.8 and 28 years, respectively) when children <5 years of age were excluded from analysis.

Ultrasonography showed prevalence of CE in the liver to be 4.7% (45/949); radiography showed prevalence of CE in the chest to be 1.1% (9/829). Two persons had cysts in the liver and lungs. Therefore, the CE prevalence according to ultrasonography and radiography was 5.5% (52/949; 95% confidence interval [CI] 4.1%–7.1%). Seropositivity according to IBCF was 8.9% (83/929; 95% CI 7.2%–10.9%); seropositivity according to rEpC1-GST was significantly higher at 19.7% (184/929; 95% CI 17.2%–22.4%, p<0.01). All 83 IBCF-positive persons reacted to the 16-kDa band; the bands of 21 and 8 kDa were observed for 78 and 76 persons, respectively. No differences were found among the 9 communities (Table 1). When participants were divided into 2 groups according to median age (≤23 and >23 years), no difference was found for proportion of those who were positive by serologic assay and by radiographic examination; however, a significant difference existed according to ultrasonographic examination (14/456 [3.1%] for those ≤23 years vs. 31/493 [6.3%] for those >23 years; p<0.05).

The total number of liver cysts was 50 (5 persons had 2 liver cysts each); the total number of lung cysts was 10 in 9 persons. The lung-to-liver ratio was 1:5. Most of the liver cysts were classified as CE5 (54%, 27/50), inactive cysts with calcified walls, followed by CE1 (20%, 10/50) active cysts and CE2 (10%, 5/50). Types CE3 and CE4 with signs of initial degeneration (8% each, 4/50) were rare (Figure 2). The average age was similar for persons with CE1, CE4, and CE5 at 40.6, 40.9, and 40 years of age, respectively. Those with CE2 and CE3 averaged 28.8 and 23.3 years of age, respectively.

The frequency of persons in the group that had CE-positive imaging results who also had IBCF-positive results was 47.1% (8/17; 95% CI 23%–72.2%) for hepatic noncalcified cysts, 35.7% (15/42; 95% CI 21.6%–52%) when hepatic calcified cysts were included, and 22.2% (2/9; 95% CI 2.8%–60%) for pulmonary cysts. Of the 52 persons who had CE

Table 1. Immunoblot assay results for cystic echinococcosis, central Peruvian Highlands, using 2 immunoblot assays with different antigens*

Community	No. samples	Antigen, no. (%) positive	
		IBCF	rEpC1-GST
Tambochaca	62	2 (3.2)	17 (27.4)
Huarautambo	54	2 (3.7)	10 (18.5)
Astobamba	79	5 (6.3)	12 (15.2)
Santiago Pampa	213	17 (8)	35 (16.4)
12 de Octubre	84	11 (13.1)	10 (11.9)
Andachaca	118	11 (9.3)	25 (21.2)
Uchumarca	113	15 (13.3)	27 (23.9)
Tambopampa	107	10 (9.4)	30 (28)
Ayayog	74	6 (8.1)	14 (18.9)
Other†	25	4 (16)	3 (12)
Total	929	83 (8.9)‡	183 (19.7)‡

*IBCF, antigen was bovine hydatid cyst fluid; rEpC1-GST, antigen was recombinant EpC1 glutathione S-transferase.
†Group of volunteers who came from nearby communities and were also included in the survey.
‡Statistically different (p<0.01), with higher percentage of seropositivity for rEpC1-GST than IBCF.

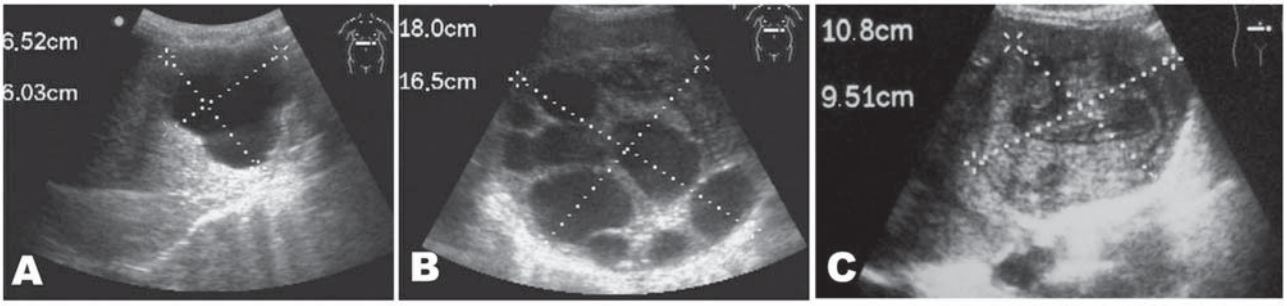

Figure 2. Ultrasonographic images of cystic echinococcosis in the liver in patients from the Yanahuanca district, Central Peruvian Highlands. A) Cyst type CE1; B) Cyst type CE2; C) Cyst type CE4.

in liver, lungs, or both, 49 provided serum samples (3 who had positive ultrasonography results did not provide a blood sample). The frequency of persons in the group that had CE-positive imaging results who also had rEpC1-GST–positive results was 16.7% (7/42; 95% CI 7%–31.3%) for all hepatic cysts, and 29.4% (5/17; 95% CI 10.3%–56%) when hepatic calcified cysts were excluded. This test detected 33.3% (3/9; 95% CI 7.5%–70%) of those with CE-positive chest radiographs. Neither serologic test detected >50% of persons with presumptive CE imaging results (Table 2). However, the IBCF was >2× as sensitive as the rEpC1 for detecting persons with CE-positive imaging results.

Of the 2 persons who had cysts in lung and liver, 1 had positive results for both serologic tests and the other had negative results for both. The frequency of seropositivity for persons with liver cysts ≤20 mm in diameter was 25% (6/24) for IBCF and 4% (1/24) for rEpC1-GST. In contrast, the frequency for persons with liver cysts >20 mm in diameter was 50% (9/18) for IBCF and 33% (6/18) for rEpC1-GST. A significant difference in detecting cysts with diameters ≤20 mm versus >20 mm was found for only rEpC1-GST (4%– 33%, $p<0.05$).

The agreement between IBCF and rEpC1-GST was only 8%, which would be expected by chance alone ($\kappa = 0.08$, $p<0.01$). Of 928 participants, 26 were positive by both serologic tests (2.8%), and 688 (74.1%) were negative by both. A total of 157 persons who had IBCF-negative results had rEpC1-GST–positive results; 57 who had IBCF-positive results had rEpC1-GST–negative results. CE-test positivity was not significantly associated with sex according to any diagnostic test (Table 3).

To assess possible cross-reactions with cysticercosis, we evaluated serum by using a purified glycoprotein *Taenia solium* antigen, in an immunoblot format (*18*). At the population level, antibodies to *T. solium* cysticercosis did not affect hydatid serologic assay results. The seroprevalence of cysticercosis was statistically similar for hydatid-seropositive and hydatid-seronegative persons, regardless of the antigen used to diagnose hydatidosis (IBCF: 11/83, 13.3% vs. 103/852, 12.1%, respectively; rEpC1-GST: 24/184, 13% vs. 90/750, 12%, respectively). Similarly, at the population level, the seroprevalence of *E. granulosus* antibodies was similar for cysticercosis-seropositive and cysticercosis-seronegative persons, regardless of the antigen used to detect *Echinococcus* (IBCF: 11/114, 9.7% vs. 72/821, 8.8%, respectively; rEpC1-GST: 24/114, 21.1% vs. 160/820, 19.5%, respectively).

Discussion

This study demonstrates and confirms the high prevalence of CE in humans in the central Peruvian Highlands. It also highlights the limited performance of 2 immunoblot tests (IBCF and rEpC1-GST) under field conditions by detecting <50% of persons who had CE-positive imaging results. We show the utility of ultrasonography for CE screening, which demonstrated an elevated percentage (54%) of apparently inactive and calcified hepatic cysts.

The survey was well accepted by the study population. However, most of the participants were women; men tend to be more reluctant to participate in medical studies, especially those involving blood sampling. The CE prevalence of 5.5% found by ultrasonography and radiography is

Table 2. Frequency of seropositive results among persons with cystic echinococcosis–positive ultrasonography and radiography imaging results, central Peruvian Highlands*

	Ultrasonography,† % (95% CI)			Overall,§ % (95% CI)	
Antigen	With calcification	Without calcification	Radiography‡	With calcification	Without calcification
IBCF	35.7 (21.6–52)	47.1 (23–72.2)	22.2 (2.8–60)	32.7 (20–47.5)	34.6 (17.2–55.7)
rEpC1-GST	16.7 (7–31.3)	29.4 (10.3–56)	33.3 (7.5–70)	18.4 (8.8–32)	26.9 (11.6–47.8)

*CI, confidence interval; IBCF, antigen was bovine hydatid cyst fluid; rEpC1-GST, antigen was recombinant EpC1 glutathione S-transferase.
†Frequency of positive abdominal ultrasonographic results.
‡Frequency of positive chest radiographic results.
§Ultrasonography, radiography, or both.

Table 3. Echinococcosis-positive results, by sex, 4 diagnostic tests, central Peruvian Highlands*

Test	Female		Male	
	No. (%) positive	Total	No. (%) positive	Total
Ultrasonography†	30 (5.2)	572	15 (4.0)	377
Radiography‡	7 (1.4)	497	2 (0.6)	332
IBCF§	54 (9.7)	558	29 (7.8)	371
rEpC1-GST¶	113 (20.3)	558	70 (18.8)	371

*By χ^2 analysis, none of the tests showed a significant association between sexes with positive diagnosis.
†Abdomen.
‡Chest.
§IBCF, antigen was bovine hydatid cyst fluid.
¶rEpC1-GST, antigen was recombinant EpC1 glutathione S-transferase.

similar to previously reported rates from other central highland communities in Peru (4.9%–5.7%) (*12*,*14*). However, a study in Vichaycocha (north highland) showed a rate of 9.3% by ultrasonography and radiography, and a seropositivity rate of 18.2% (*11*). These rates are comparable to the highest reported prevalences in other countries such as China (liver CE from 3.3% to 6.6% [*22*,*23*]), Kenya (5.6% liver CE [*1*]), and Argentina before initiation of its control program (5.6% in school children [*24*]).

Unlike some other areas of Peru, Yanahuanca has not had a CE control program. This might explain, at least in part, why the overall prevalence in our survey was as high as 12.5% when ultrasonography, radiography, and IBCF results were combined and up to 23.8% when ultrasonography, radiography, and rEpC1-GST results were combined. Notwithstanding, these figures do not represent the true disease prevalence because they might reflect the continuous transmission and endemicity of *E. granulosus* in this region. Diagnostic approaches for CE based on imaging techniques can be problematic because of variations in size, shape, and location of the cysts. In addition, *E. granulosus* distribution, host susceptibility, and strain variation might affect disease transmission in different areas of the Peruvian Highlands (to our knowledge, no studies have tried to characterize *E. granulosus* strains in Peru).

One of the underlying weaknesses of this study was the lack of a true standard (a test with 100% sensitivity and 100% specificity), which would enable evaluation of alternative diagnostic tests and underlying prevalence. Most areas of medicine lack a true standard, yet recent statistical techniques have been developed that can help evaluate diagnostic tests and estimate true prevalence in the absence of such a standard. Most of these techniques rely upon a Bayesian framework (*25*–*27*) and are computationally intensive but more flexible than maximum likelihood–based approaches because they can incorporate correlation among diagnostic tests. Although the Bayesian approach offers distinct advantages, potential problems include specification of an appropriate prior distribution and a nonidentifiable model. Our study encountered both of these problems because reliable prior information is not readily available and estimates provided by a Bayesian approach are limited by the lack of identifiable groups.

The poor performances of the IBCF and rEpC1-GST testing may be related to false-positive imaging results from other space-occupying lesions (e.g., neoplasia, abscesses, nonparasitic cysts). Additionally, participants might have had low or undetectable levels of circulating antibodies from different stages of cyst development or degeneration. The production of IgG depends on the number, size, location, and condition of the cysts; only 60%–80% of persons with confirmed CE become seropositive (*28*). Calcified cysts are less seroreactive, thus decreasing seropositivity (*16*,*22*), as observed in this study. Previous studies using the same IBCF testing found that the frequency of seropositivity was 57% (*12*) and 53% (*14*) for liver hydatid cysts (ultrasonography), and 13% for lung cysts (*11*), similar to what we found in this study. However, another study, in which most of the liver cysts were active, reported a proportion of persons who were positive according to IBCF testing to be as high as 73% (*11*).

Other possible reasons for the limitation of serologic testing might be the weak immune response against pulmonary cysts, cysts at other sites (e.g., brain, eyes, bones, ovaries), small or poorly defined cysts, and a thick collagen cyst wall that would reduce antigen exposure (*29*). However, increased seropositivity (up to 50%) for detecting large hepatic cysts (>20 mm in diameter) has not been reported previously and may be due to elevated antigen concentrations in these cysts. To our knowledge, the only study showing a correlation between cyst size and seropositivity was performed in sheep (*30*).

The different antigen sources would explain the disparity and poor agreement between the 2 serologic tests. EpC1 is a recombinant antigen obtained from protoscolex larvae from sheep hydatid cysts (*17*), while the IBCF uses bovine hydatid cyst fluid (*16*). The IBCF appears to be more responsive than the rEpC1-GST in detecting CE-image cases. Crude hydatid fluid has been recommended for mass serologic screening (*31*) and purified antigen 5 (Ag5) and AgB for specific diagnosis. Ag5 and AgB are recognized as 2 of the most useful *E. granulosus* antigens for diagnosis (*32*), although Lorenzo et al. (*33*) found that hydatid cyst fluid, AgB, and its subunit AgB8/1 exhibited equivalent diagnostic efficiencies in a randomized multicenter study.

Among participants with CE-negative ultrasonography images, rEpC1-GST testing detected >3× more seropositive persons than the IBCF (19% vs. 7.3%, respectively). This scenario has been described with other diagnostic test such as AgB ELISA, which detected 5.3% CE-seropositive persons in a group with CE-negative ultrasonography images (*22*). We do not have evidence of cross-reaction with

Fasciola for any of the serologic tests used in this study; however, *Hymenolepis nana, Entamoeba histolytica, Giardia lamblia,* and *Taenia sp* (*34*) are endemic to the study area, which might affect the serodiagnosis. We demonstrated no cross-reaction with cysticercosis (*T. solium*) by IBCF or rEpC1-GST because the proportion of persons who were cysticercosis positive was equal among those who were CE seropositive and CE seronegative according to both assays. Other possible explanations include past exposure to *Echinococcus* eggs (aborted infection) that produced only transient antibodies (*35*), and undetected cysts.

In our study, the lung-to-liver ratio of 1:5 is higher than ratios reported in other epidemiologic studies in Peru (*11*) but within the range of those reported in other *Echinococcus*-endemic South American countries such as Argentina, Chile, and Uruguay (1:3–1:13) (*11*,*36*). Molecular genetic studies of *E. granulosus* in the Peruvian Highlands may clarify some issues about the organ infection preferences (tropism), tissue survival, infection rates, immune responses, and the performance and agreement of immunodiagnostic tests.

Among liver cysts, 54% were CE5 and 20% were CE1; other studies have typically displayed an exponential decline in the frequency of liver cyst types from CE1 (most frequent) to CE5 (most rare) (*37*). Because most of these persons had not received a diagnosis of hydatid disease or antihelminthic treatment, these calcified forms are most probably the result of the natural process of degeneration driven by individual immune responses (*38*). A proportion of hydatid cysts die after initial establishment; thus, calcified lesions can be observed macroscopically (*29*). The geographic variation of cyst type frequencies can also depend on the time between infection and evaluation, the immune response of a highly exposed population, and *E. granulosus* genetic variation (*15*). Calcified cysts may also have been misdiagnosed with other lesions from biliary cysts, pyogenic abscesses, amebic liver abscesses, or even tumor-like masses or metastases (*39*), although these conditions are uncommon in Peru. We did not study the specific IgG subclasses in relation to cyst types, but their quantification may be important for understanding the natural history of hydatid cyst. IgG_4 antibody response is associated with development, growth, and progression (CE1 to CE3); IgG_1, IgG_2, and IgG_3 occur predominantly when cysts became infiltrated or degenerated (CE4 and CE5) (*40*).

Our study provides data on CE in the surveyed communities and shows the results of using ultrasonography, radiography, and immunodiagnosis for large-scale population screening. Determining baseline prevalence with ultrasonography enables the evaluation of epidemiologic surveillance activities and study of the natural history of CE. Ultrasonography is well accepted by the population and is relatively less expensive than other imaging techniques. Methods that are inexpensive and relatively easy to use, such as immunodiagnosis and ultrasonography, are required for large-scale screening of populations in which hydatidosis is endemic. However, serologic assays have serious limitations under field conditions, as has been demonstrated in this study. Seroepidemiologic surveys for CE require better diagnostic antigens and should be supported by imaging methods whenever possible.

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Cost-effectiveness of Antiviral Stockpiling and Near-Patient Testing for Potential Influenza Pandemic

M. Ruby Siddiqui* and W. John Edmunds*

A decision analytical model was developed to investigate the cost-effectiveness of stockpiling antiviral (AV) drugs for a potential influenza pandemic in the United Kingdom and the possible role of near-patient testing in conserving AV drug stocks. Under base-case assumptions (including a fixed stockpile that was smaller than the clinical attack rate), the treat-only option (treating all symptomatic patients with AV drugs) would be considered cost-effective (£1,900–£13,700 per quality-adjusted life year [QALY] gained, depending on the fatality scenario), compared with no intervention (nonintervention but management of cases as they arise). The test-treat option (testing all symptomatic patients but treating those with positive tests results only) would result in moderate gains in QALYs over the treat-only option but at relatively large additional costs. Stockpiling sufficient AV drugs (but not near-patient tests) to treat all patients with clinical cases would be cost-effective, provided AV drugs are effective at preventing deaths from pandemic influenza.

Many countries are ordering stockpiles of antiviral (AV) drugs for use in a potential influenza pandemic. The United Kingdom recently announced the procurement of 14.6 million courses of oseltamivir, enough for almost 25% of the population (*1*).

The timing of an influenza pandemic cannot be predicted (the most recent pandemics occurred in 1918, 1957, and 1968/69). Because AV drugs have a limited shelf-life, long-term maintenance of stockpiles may constitute a significant cost. Similarly, the size (clinical attack rate [CAR]) of a pandemic cannot be accurately foreseen. Therefore, triaging of patients with influenza-like illness (ILI) may be essential to conserve limited AV drug stocks. Possible triaging methods include near-patient testing (rapid diagnostic tests at the point of care). This study assessed the cost-effectiveness of stockpiling AV drugs for a potential influenza pandemic and, in the event of a pandemic, also assessed the use of near-patient testing in the management of AV drugs.

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Methods

A decision analytical model (Figure 1) was constructed to compare the costs and quality-adjusted life year (QALY) loss associated with 3 potential strategies for the management of patients with ILI in the United Kingdom: 1) do not treat with AV drugs and manage complications if they arise (no intervention), 2) treat all patients with AV drugs (treat only), or 3) test then treat those who test positive for influenza with AV drugs (test-treat). Precision Tree (Palisade Corporation, Ithaca, NY, USA) running in Microsoft (Redmond, WA, USA) Excel was used to construct the model and @Risk (Palisade Corporation) was used to perform the probabilistic sensitivity analysis.

Epidemiologic Scenarios

Baseline epidemiologic scenarios were based on the UK Department of Health Pandemic Contingency Plan (*2*), which assumes a cumulative CAR of 25% over 1 wave lasting 15 weeks. The demand on general practitioners (GPs) and accident and emergency departments (A&Es) would require an alternative means for AV drug distribution (*2*). Patients with ILI were therefore assumed to have received AV drug therapy by visiting teams or call-in centers. Of these, because of secondary complications, 5% were assumed to have consulted further with a GP and 5% with A&E departments. Also, among ILI patients, 0.55% were expected to be hospitalized. All model parameters are summarized in the online Appendix Table (available from www.cdc.gov/EID/content/14/2/267-appT.htm).

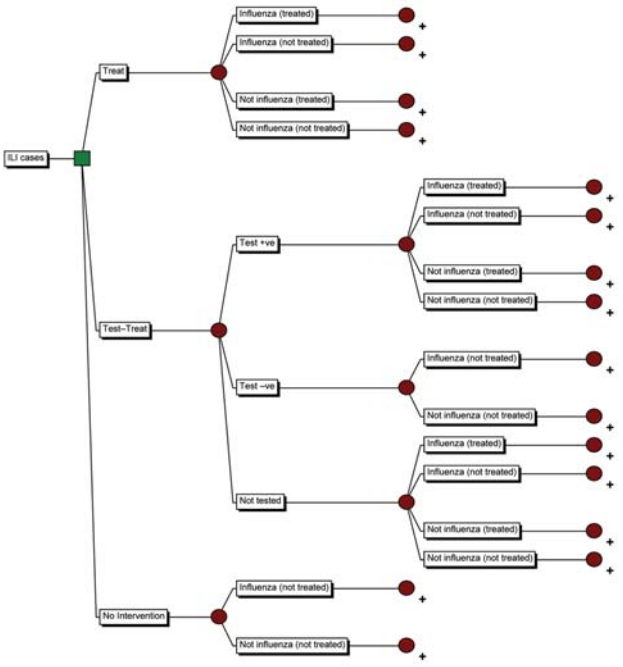

Figure 1. Decision analytical model tree of treatment strategies for patients with an influenza-like illness (ILI) during an influenza pandemic. All branches culminate in the subtree (indicated with +). QALY, quality-adjusted life year; CAR, clinical attack rate.

Background ILI rates were deduced from the mean ILI consultation rates in England and Wales observed by GP-based sentinel surveillance from 1985–2003 (*4*) and the estimated proportion (28%) of clinical ILI case-patients who consulted with GPs in interpandemic periods (*5*). Epidemic influenza is seasonal with a higher incidence in winter (wk 40–12) than summer (wk 13–39). The mean weekly ILI incidence was assumed to have remained unchanged during the pandemic, resulting in a cumulative incidence of 3.1% (winter) or 1% (summer). An additional value of 4% for midwinter (wk 49–8) was also considered in the sensitivity analyses. It is possible that a pandemic strain would out-compete the existing epidemic influenza strains and the background ILI rate would fall. However, other pathogens (e.g., respiratory syncytial virus and rhinovirus) that contribute to ILI would remain unaffected (*22*). The background ILI rate was therefore assumed to remain unaltered. The base-case analysis assumed a winter pandemic, giving a probability (PrF) of 89% (CAR/(CAR + background ILI rate) that a case of ILI was pandemic influenza .

Two fatality scenarios were assumed, on the basis of the 1918 influenza pandemic (1918 scenario), which had an overall case-fatality ratio (CFR) of 2.3%, and the 1957 and 1968/69 influenza pandemics (1957/69 scenario) which had an average CFR of 0.3%, with most deaths occurring in the elderly (online Appendix Table).

Three pandemics occurred in the 20th century, which suggests that a pandemic occurs approximately every 30 years but that this is a random (Poisson) process. For the base-case analysis, we assumed that the next pandemic would take place in 30 years; this figure was varied from 1 to 50 years in the sensitivity analyses.

Antiviral Treatments

The neuraminidase inhibitor oseltamivir was selected as the AV treatment of choice because it is cheaper and easier to stockpile than zanamivir and has been shown to dominate zanamivir in cost-utility analyses (*23*). Cyclic amines (amantadine and rimantadine) were not considered because resistance to these drugs has emerged in influenza A virus (H5N1) with pandemic potential (*24*). The rate of adverse events after the use of neuraminidase inhibitors is low and therefore was not considered in this analysis (*12*).

Meta-analyses of oseltamivir efficacy studies were compared, and the 2003 study by Kaiser et al. was selected for the parameterization of this model because it included the greatest number of subjects (*5,15,20*). No data were available for AV efficacy in reducing influenza-related deaths so the same efficacy as that for reducing influenza-related hospitalizations was assumed. Assuming all GP and accident and emergency (A&E) consultations were attributable to the development of complications (*2*), the probability of complications and the probability of hospitalizations (given no AV therapy) were calculated by using the odds ratios from the oseltamivir meta-analyses (*15*). The probability of complications for noninfluenza ILI patients was considered the same as for untreated influenza patients, but the probability of hospitalization for noninfluenza ILI patients was considered to be 40% that of untreated influenza patients (*15*).

We assumed that for both the treat-only and test-treat strategies all qualifying patients were given AV drugs and were tested until the stockpile ran out. No epidemiologic differences were assumed between those who received AV drugs and those who did not because the stockpile was depleted. Neuraminidase inhibitors are only recommended for use within 48 hours of symptom onset (*25*). We assumed that the efficiency of AV drug distribution would be such that 70% of ILI patients would receive timely AV drugs; this varied from 30% to 97% in our sensitivity analyses (online Appendix Table). Those receiving AV drugs after 48 hours were assumed to derive no benefit.

The shelf-life of oseltamivir is currently 5 years. However, tests are being conducted to increase this to 6 years (*6*). Shelf-life was varied in our sensitivity analyses between 4 and 6 years.

For base-case analyses, we assumed the AV drug stockpile to be 14.6 million courses (*1*), giving an 87% probability of case-patients receiving AV drugs (size of

Table. Total NHS costs and QALY loss (discounted at 3.5%) resulting from an influenza pandemic occurring in 30 years assuming 1918 or 1957/69 CFR*

Treatment program	Pandemic influenza cases (millions)	Pandemic influenza deaths (millions)	Discounted NHS costs (million £)	Discounted QALY loss (millions)	Incremental cost per QALY (£)
1918 scenario					
No intervention	15	0.344	113	2.23	
Treat only	15	0.236	1,361	1.56	1,861†
Test-treat	15	0.231	2,356	1.53	31,031‡
1957–69 scenario					
No intervention	15	0.044	113	0.395	
Treat only	15	0.030	1,361	0.303	13,668†
Test-treat	15	0.030	2,356	0.299	227,896‡

*NHS, National Health Service; QALY, quality-adjusted life year; CFR, case-fatality ratio.
†Cost per QALY gained over no intervention program.
‡Cost per QALY gained over treat-only program.

stockpile/total ILI cases). Hence, the size of the AV stockpile would be limited under the treat-only strategy.

Near-Patient Tests

A number of near-patient influenza tests are currently available (*7–10*). To reflect the continual improvement in test technology, a composite test was constructed based on the best performance of currently available tests (89.5% sensitivity and 99.8% specificity) (Directigen Flu A+B, Becton Dickinson, Sparks, MD, USA) (7,*12*), a shelf-life of 2 years (Quickvue, Quidel Corporation, San Diego, CA,USA) (*9*), and a cost of £7 per test (Biostar Flu OIA, Inverness Medical-Biostar Inc, Louisville, CO, USA) (*8*). Parameter distributions for test sensitivity and specificity were deduced from a meta-analysis of near-patient tests (*12*), but distributions were assumed for the shelf-life and cost.

Although the United Kingdom has not accumulated a stockpile of near-patient tests, we assumed it to be the same size as the AV drug stockpile in the base-case analysis. The probability of being tested (test-treat option) was 87% (size of stockpile/total ILI cases).

Costs

As recommended in the United Kingdom, costs were analyzed from the perspective of the healthcare provider, the National Heath Service (NHS). Future costs and benefits were discounted at the current rate of 3.5% per annum and all costs were in 2004 pounds sterling (£1 = ≈US$1.8) (*14,26*).

Mean unit costs per GP consultation**,** hospitalization, and A&E attendance for ILI were deduced from standard sources (*17,19*). We assumed that no additional costs were associated with death. The costs of complications leading to GP consultation or hospitalization were assumed to be the same for pandemic influenza and nonpandemic ILI.

The unit cost of an AV treatment course was assumed to be £16 (*16*) for the treat-only option and £16.87 (because of excess AV drugs) for the test-treat option (fixed stockpiles). The unit cost of a near-patient test was assumed to be £7 (*7*), and storage was £1 per unit (AV drug course or test) per year for both programs. Administration costs for the distribution of AV drugs or near-patient testing by visiting teams or call-in centers were assumed equivalent to the mean cost of a home visit by a district nurse, health visitor, health care assistant, or practice nurse (£15.75 per test or AV drug course) (*17*). Units were assumed to be procured >2 years (*1*) and replenished at expiration of shelf-life.

Health Benefits

Health benefits were assessed by using QALYs. QALY loss associated with uncomplicated ILI were calculated study by O'Brien et al. (*20*) assuming a normal health score of 0.85 (online Appendix Table). QALY loss associated with the development of complications was assumed to be the sum of QALY loss associated with uncomplicated ILI and that associated with pneumococcal pneumonia (outpatient) because this was the most likely complication (*21*). Similarly, QALY loss associated with hospitalization was considered the sum of QALY loss associated with uncomplicated ILI and that associated with pneumococcal pneumonia (inpatient) (*21*).

The mean discounted QALY loss associated with pandemic influenza death was estimated by using age-specific CFR under the 2 death scenarios and background life expectancy by age weighed by age-adjusted quality-of-life scores (online Appendix Table) (*27*). QALY loss associated with noninfluenza death was assumed to be the same as that associated with *S. pneumoniae* death (a mean discounted QALY loss per noninfluenza death of 6.09 from 1980–2000) (*3*).

Base-Case Assumptions

In the base-case analysis, the cost-effectiveness of the potential strategies was compared under the assumptions of fixed AV and test stockpiles (14.6 million units), a CAR of 25%, and a time to pandemic of 30 years. There is no threshold for cost-effectiveness in the United Kingdom, although the National Institute for Clinical Excellence will probably

reject an intervention on cost-effectiveness grounds if the cost per QALY gained is in excess of £25,000–£35,000 (*28*). For ease of exposition, we used a simple threshold of £30,000 per QALY to define a cost-effective intervention.

Results

Base-Case Analysis (Fixed Stockpiles)

In our base-case model, we estimated that in a pandemic, ILI would develop in 28.1% of the population (16.8 million persons) (including 15 million pandemic influenza patients). Under the high CFR conditions of the 1918 scenario, ≈344,000 deaths would occur compared with ≈44,000 deaths under the 1957/69 scenario (Table). These scenarios would result in the loss of ≈2.2 or 0.4 million discounted QALYs, respectively, with a total discounted cost to the NHS of £113 million if no treatment program were initiated (no intervention). The treat-only program would reduce this loss by 700,000 or 90,000 QALYs at a cost of ≈£1,900 or £13,700 per QALY gained for the 1918 and 1957/69 scenarios, respectively, well below the £30,000 threshold. The test-treat program would further reduce this loss slightly by 30,000 or 4,000 QALYs but at a high cost of ≈£31,000 or £228,000 per QALY gained over the treat-only alternative. The test-treat option would be unlikely to be considered because cost-effectiveness is highly dependent on the fatality scenario.

Univariate Sensitivity Analysis of the Treat-Only Program (Fixed Stockpile)

Because the treat-only program was the most cost-effective program under both fatality scenarios, we carried out a univariate sensitivity analysis of the incremental cost-effectiveness of this program to variability in model parameters (online Appendix Table). AV drug efficacy for reducing complications and hospitalizations had minimal effect on the cost-effectiveness of the treat-only program, but this strategy was highly sensitive to AV drug efficacy for reducing death (Figure 2). This was due to the relatively high QALY loss associated with pandemic influenza death (94% and 69% of the total QALY loss for the 1918 and 1957/69 scenarios, respectively). Because the value of this parameter is unclear, further studies of the potential protective effect of AV drugs against death are essential.

The timing of the pandemic and the discount rate were influential parameters. However, variation in the timing of the epidemic is unlikely to change the recommendation that the treat-only strategy is cost-effective. If an epidemic occurs in 45 years, the costs per QALY gained would be ≈£3,800 and £28,000 for the 2 fatality scenarios (discount rate of 3.5%), still below the £30,000 threshold. At a discount rate of 6%, the treat-only option would be cost-effective for up to 30 years.

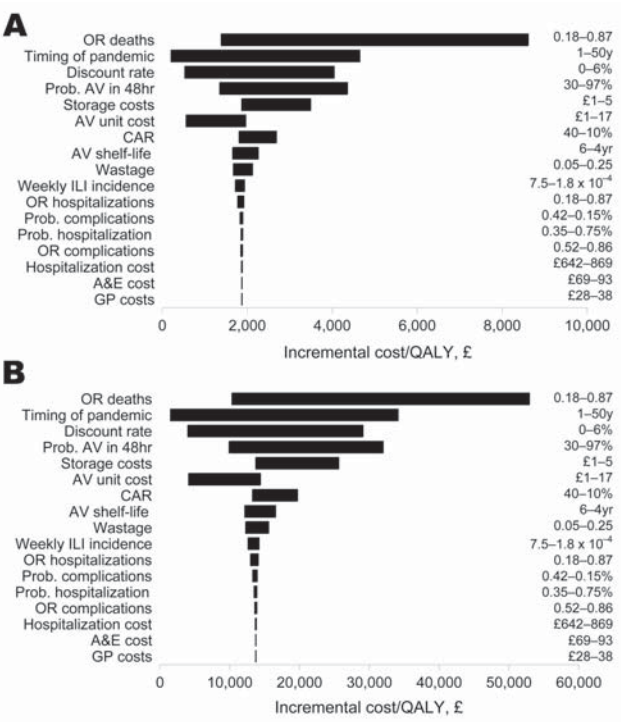

Figure 2. Univariate sensitivity analyses of the incremental cost-effectiveness of the treat only strategy over the no-intervention strategy to model parameters under the 1918 scenario (A) and 1957/69 scenario (B). OR, odds ratio; AV, antiviral; CAR, clinical attack rate; ILI, influenza-like illness; A&E, accident and emergency department; GP, general practitioner; QALY, quality-adjusted life year.

The efficiency of AV drug distribution is likely to be important. The program would however remain cost-effective if the probability of receiving AVs within 48 hours did not drop below 35% (£3,700 or £27,000 per QALY gained for the 1918 and 1957/69 scenarios), respectively.

The treat-only program was slightly more cost-effective in the summer than the winter or midwinter as the probability of pandemic influenza being the cause of ILI was higher (96%, 89%, and 84%, respectively). Wastage (wasted quantities, fraud, theft) of AV drug supplies as high as 25% had little effect on cost-effectiveness of the treat-only option (Figure 2).

A lower CAR (15%) reduced the cost-effectiveness of AV drugs because some of the stockpile would not be used (surplus). Increasing the CAR above 25% had no effect on programs with fixed stockpiles because the same number of deaths and complications would be prevented at the same cost (the proportion of deaths prevented would be reduced, but the absolute number would remain the same).

Threshold Conditions for Test-Treat Option (Fixed Stockpiles)

For high CARs, where a fixed AV drug stockpile is less than the expected demand (as in the base-case), near-pa-

tient tests could be used to better target therapeutic courses. A univariate sensitivity analysis of the incremental cost-effectiveness of test-treat over treat only to variability in the near-patient test parameters, test sensitivity, specificity, unit cost, and shelf-life, was carried out. Under the 1918 scenario the test-treat strategy would require test sensitivity to exceed ≈90% (Figure 3, panel A) or a test unit cost below £6 or a shelf-life above 3 years (Figure 3, panel B) to be considered cost-effective. Test specificity would have little effect on the incremental cost-effectiveness because it has no effect on QALY loss. Under the 1957/69 scenario test-treat would never cross the cost-effectiveness threshold even with a 100%-sensitive or 100%-specific test, a test cost as low as £0, or a shelf-life as high as 4 years.

Probabilistic Sensitivity Analysis (Fixed Stockpiles)

Model parameters were varied (online Appendix Table) in a probabilistic sensitivity analysis, which suggests that for fixed AV drug and test stockpiles, the probability is high that the treat-only option would be cost-effective, irrespective of the fatality scenario (Figure 4). The test-treat option would result in small QALY gains (and often losses) but at substantial additional costs. The probability of this strategy being cost-effective is low compared with the treat-only option, particularly for the 1957/69 fatality scenario.

Incremental Cost-effectiveness during a Pandemic Wave (Fixed Stockpiles)

The probability that an ILI case will be due to pandemic influenza will vary over the time course of a pandemic (assumed to peak between wk 6 and 7 for a wave lasting 15 wk) (*2*). Therefore, near-patient testing may be useful during early stages of a pandemic when clinical judgment is low and inappropriate AV administration is high. The cost-effectiveness of test-treat over a pandemic wave was analyzed.

The AV drug stockpile was assumed to remain fixed at 14.6 million courses (*1*), and the test stockpile was varied with the cumulative number of ILI cases expected per week of the pandemic wave. Figure 5 shows the total incremental cost-effectiveness of the test-treat strategy over treat only for each test stockpile for a CAR of 25%. Test-treat would be cost-effective (<£30,000 per QALY gained) for test stockpiles up to 12.1 million (the expected no. of cumulative ILI cases at wk 8 of a pandemic) under the 1918 scenario. Test-treat may even be considered for test stockpiles up to 13.7 million (wk 9 of a pandemic) as the cost-effectiveness was ≈£32,700 per QALY gained. However, under the 1957/69 scenario test-treat would not be cost-effective at any stage of the pandemic, although it may be considered for test stockpiles up to ≈35,000 (wk 2 of a pandemic) as the cost was ≈£34,000 per QALY gained.

For a CAR of 15% (data not shown), test-treat would not be cost-effective throughout a pandemic as the AV drug stockpile would exceed demand (cumulative ILI cases). For a CAR of 35% (data not shown), although test-treat would be cost-effective for test stockpiles up to 16.4 million (wk 8) under the 1918 scenario, it would not be cost-effective at any stage of the pandemic under the 1957/69 scenario. Therefore in the short-term, stockpiling enough tests for the first 2 weeks of a potential influenza pandemic (≈35,000 tests) could help conserve limited AV drug stockpiles. However, this is highly dependent on the CAR and CFR.

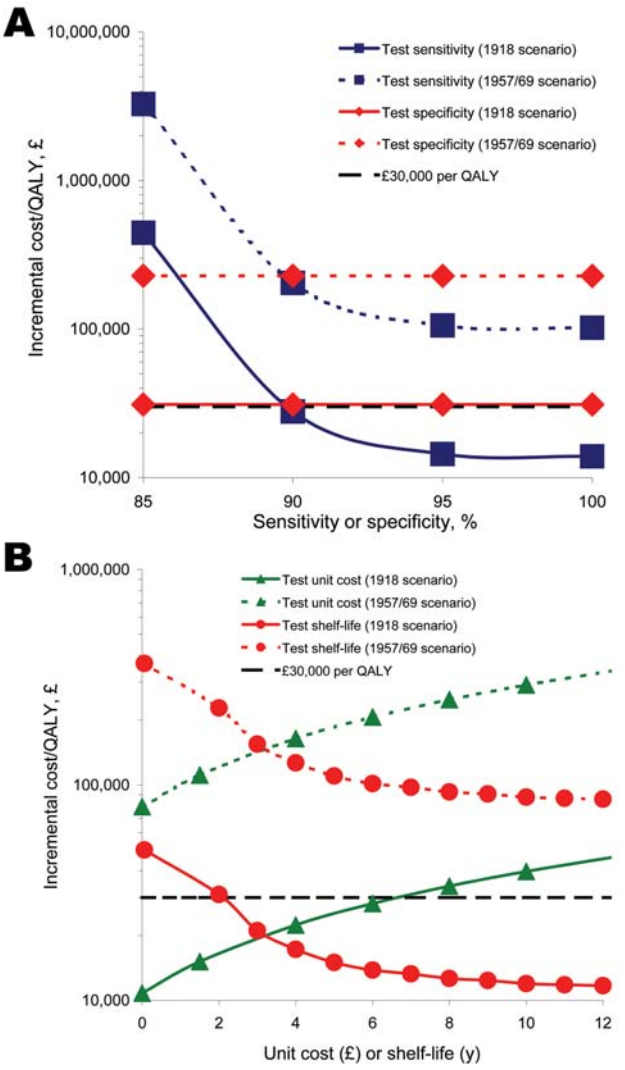

Figure 3. Univariate sensitivity analyses of the incremental cost-effectiveness of the test-treat strategy over the treat only strategy to A) near-test sensitivity and specificity and B) near-test unit cost and shelf-life. The test-treat program becomes cost-effective below the cost-effectiveness threshold (£30,000 per quality-adjusted life year [QALY] gained).

Optimal Stockpiling

In the long term, it may be more cost-effective to increase stockpiles to cover expected demand (CAR +

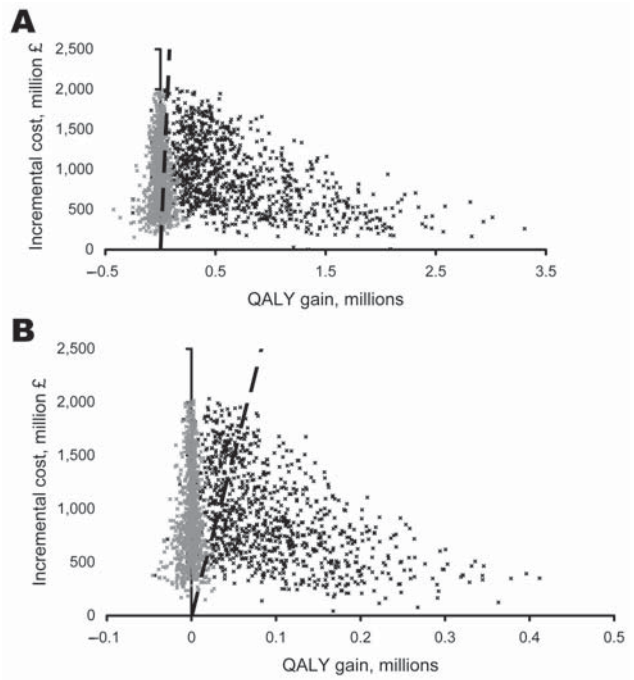

Figure 4. Probabilistic sensitivity analysis of the incremental cost-effectiveness of the treat-only over the no-intervention strategy and the test-treat strategy over the treat-only strategy for the A) 1918 and B) 1957/69 death scenarios (1,000 iterations). Cost-effective strategies lie to the right of the cost-effectiveness threshold (£30,000 per quality-adjusted life year [QALY] gained).

background ILI + wastage). Figure 6 and the online Appendix Figure (available from www.cdc.gov/EID/content/14/2/267-appG.htm) show the expected costs and QALY losses under a range of different AV drug and test stockpiles and CARs (base-case test characteristics and AV drug efficacy assumed). Each point represents 1 scenario (test and AV drug stockpile size). Points on the efficiency line were potentially cost-effective strategies. Strategies that increase cost but reduce QALY loss (moving from left to right on the efficiency line) should be considered until the slope of the line exceeds the threshold of £30,000 per QALY (efficiency line ends). For each CAR, this process suggested that the optimal strategy was treat only, stockpiling enough AV drugs to meet demand (CAR plus background ILI plus AV drug wastage). Therefore, for a CAR of 25%, the optimum stockpile was ≈20 million AV drugs only (Figure 6) because the expected number of ILI cases would be 16.8 million (15 million of which would be pandemic influenza) and the expected AV drug wastage would be 2.2 million. The test-treat strategies were never on the efficiency line, even for a perfect test (100% sensitivity and specificity), because they resulted in similar QALY loss as treat only but at increased costs. Indeed, when the size of the AV drug stockpile exceeded the demand, test-treat resulted in increased QALY loss (if the test is not 100% sensitive), because some true pandemic influenza case-patients would be denied treatment even though there was a surplus of AV drug courses.

Discussion

This study demonstrates that stockpiling AV drugs for a treat-only program is likely to be a cost-effective strategy in preparation for a potential influenza pandemic, even if the pandemic occurs many years from now, assuming that AV drugs provide some protection against death. However, under current UK planning assumptions (CAR 25%), the AV stockpile would be too small (at 14.6 million courses) to treat all cases of ILI. Near-patient testing is unlikely to be a cost-effective approach to conserving AV stocks but might be considered early in a pandemic. A more cost-effective strategy would be to increase the stockpile of AV drugs. Since CARs in excess of 30% have been observed in pandemics (*29*), increasing the stockpile to cover this possibility may be both prudent and cost-effective. Indeed, expanding the stockpile of AV drugs to encompass the whole UK population (≈60 million) might even be acceptable (≈£6,500 per QALY gained over a no intervention strategy for the 1918 scenario under base-case assumptions).

Stockpiling AVs is a cost-effective option, even though some benefits have been ignored (e.g., possible reduction in CAR if widespread and prompt treatment is offered) (*30*). Furthermore, additional benefits could be derived from using AV drug stockpiles close to their expiration dates to treat epidemic influenza patients. Finally, the reduction in illness and death that may result from widespread AV drug use is likely to bring benefits to other sectors of the economy.

This study focused on mass treatment strategies because this is the policy of the United Kingdom and other

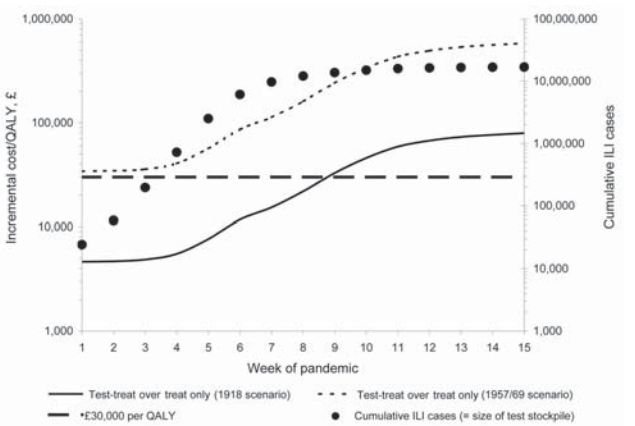

Figure 5. Incremental cost-effectiveness of the test-treat strategy over the treat-only strategy during a pandemic wave (antiviral [AV] stockpile = 14.6 million courses, test stockpile = number of cumulative influenza-like [ILI] cases, clinical attack rate = 25%). QALY, quality-adjusted life year.

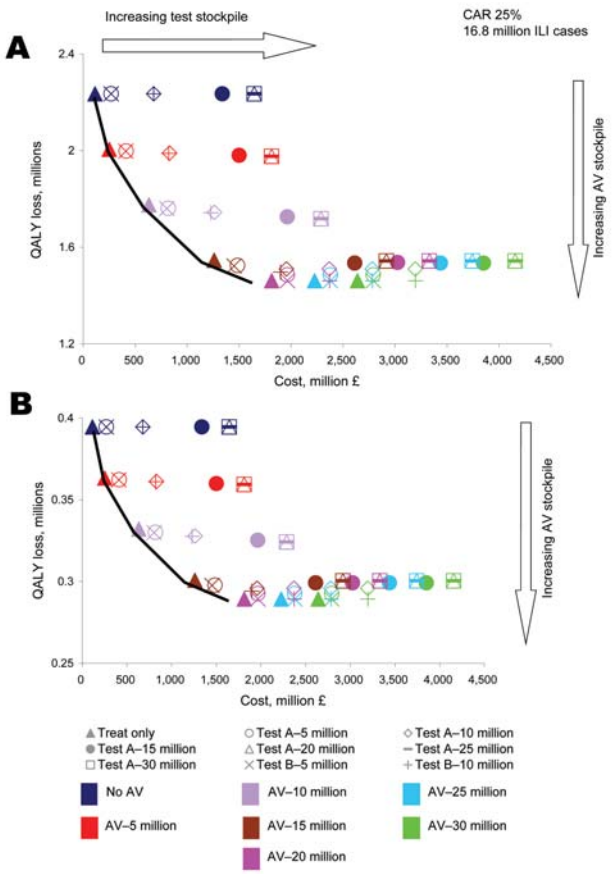

Figure 6. Optimal cost-effectiveness of antiviral (AV) and test stockpiling (0–30 million units) for a clinical attack rate (CAR) of 25% under the A) 1918 and B) 1957–69 scenarios. The composite test (Test A) and a perfect test of 100% sensitivity and 100% specificity (Test B) are included. The most cost-effective strategies lie on the efficiency line. ILI, influenza-like illness; QALY, quality-adjusted life year.

countries (*31*). However, strategies targeting those at higher risk of complications or death would be more cost-effective provided the delivery costs are similar and AV drugs are as effective in these groups.

The main caveat to an AV drug stockpiling strategy is the uncertainty concerning the efficacy of AV drugs against the next strain of pandemic influenza, particularly efficacy against influenza-related deaths. Clearly, estimation of this important parameter should be a priority if governments are to commit large resources to mitigating the effect of an uncertain pandemic occurring at an unknown point in the future.

Use of AV drugs on the scale anticipated may create a selective pressure (any factor that leads to preferential survival of organisms with specific traits) for the emergence of AV drug resistance. In this case, large-scale use of AVs could lead to the preferential survival of flu viruses that are resistant to AVs. However, studies to date indicate that resistance to oseltamivir in influenza virus strains occurs rarely (*32,33*) and that such mutations may have a fitness cost in terms of impaired growth and transmissibility (*34,35*). Nevertheless, recent reports suggest reduced susceptibility to oseltamivir in some currently circulating strains of avian influenza (H5N1) with pandemic potential (*36–38*). Clearly this may reduce the effectiveness of an oseltamivir stockpiling program, although AV drugs might still delay pandemic spread to allow vaccine development (*39*). Further epidemiologic and modeling studies of the potential effect of oseltamivir resistance on viral fitness and drug effectiveness are required.

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Streptococcus pneumoniae Serotype 19A in Children, South Korea

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Despite the concern of replacement disease, notably by serotype 19A after 7-valent conjugate vaccine (PCV7) use, serotype 19A was increasingly recognized in Korean children before the introduction of PCV7. To understand the dynamics of serogroup 19 prevalence from 1991–2006, we serotyped 538 pediatric pneumococcal isolates. Serogroup 19 isolates (n = 126) were characterized by antimicrobial drug susceptibility, presence of *mef*A/*erm*B, and multilocus sequence typing. Overall, the proportion of serotype 19A isolates increased but serotype 19F decreased. Among children <5 years of age, the proportion of serotype 19A isolates in invasive pneumococcal disease increased from 0% in 1991–1994 to 8%–10% in 1995–2000, reached 26% in 2001–2003, and remained at 20% in 2004–2006 when vaccine coverage did not exceed 25% (p = 0.005 for trend). This study demonstrates that the expansion of multidrug-resistant ST320 was responsible for the increase in serotype 19A before PCV7 use.

Streptococcus pneumoniae is a major cause of invasive infections in young infants and children. Among >90 serotypes, only a limited number account for pneumococcal diseases. Serotype incidence can vary by patient age, geographic region, and time of surveillance. Since the introduction of 7-valent conjugate vaccine (PCV7) in the United States, a decrease in the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes has been observed in pediatric and nonpediatric age groups (*1,2*). However, the incidence of IPD caused by nonvaccine serotypes (including serotype 19A) increased 1.5-fold in 2002 compared to that in 1999 (*2,3*). To date, replacement for IPD has been observed for serotypes 3, 15, 19A, 22F, 33F, and 35, with the increase in 19A being the most prevalent. (*2–7*). Recently, Singleton et al. reported that serotype 19A was responsible for 28.3% of IPD in rural Alaska Native children <2 years of age during 2004–2006 (*8*). In addition, recent studies from Massachusetts and Texas showed that a multidrug-resistant sequence type of serotype 19A has emerged as an important cause of IPD (*9,10*).

The direct effects of PCV7 in serotype distribution changes and genetic structures of pneumococcal isolates are not clear. However, capsular switching of vaccine serotypes under selective pressure by PCV7 is one of the mechanisms underlying the expansion of serotype 19A (*4,11–13*).

As part of this surveillance implemented in a tertiary referral center in South Korea, all pneumococcal isolates obtained from sterile body fluids and from various clinical specimens were subjected to serotype and antimicrobial drug susceptibility pattern determinations (*14*). Surveillance since 1991 shows that serotype 19A isolates were increasingly recognized among clinical isolates before PCV7 was introduced in South Korea in November 2003. We describe *S. pneumoniae* serotype distribution changes in Korean children during 1991–2006 with an emphasis on serogroup 19.

Methods

Patients and Pneumococcal Isolates

Hospital-wide surveillance was continued to monitor pneumococcal diseases as a part of routine clinical care at Seoul National University Children's Hospital from 1991

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through 2006. Only initial isolates were included in the study; repeated isolates from the same patient were excluded. Isolates were classified as invasive (e.g., blood, pleural fluid, cerebrospinal fluid, joint fluid) and noninvasive (pharynx, middle ear fluid, sputum). Methods of surveillance and of obtaining blood cultures did not change during the study period.

Analysis of Serotype Distributions and Classifications

All isolates were serotyped by the Quellung reaction using antiserum (Statens Serum Institute, Copenhagen, Denmark). The study period was divided into five 3- or 4-year-periods: 1991–1994 (period 1), 1995–1997 (period 2), 1998–2000 (period 3), 2001–2003 (period 4), and 2004–2006 (period 5). The PCV7 serotypes were 4, 6B, 9V, 14, 18C, 19F, and 23F. Serotypes considered to be PCV7 related included those not directly targeted by PCV7 but of the same serogroups (6A, 9A, 9N, 18B, 18F, and 23A); serotype 19A was analyzed separately. Non-PCV7 serotypes included all other serotypes.

Multilocus Squence Typing Analysis

Multilocus sequence typing (MLST) was performed on all serotype 19A isolates (n = 58) and serotype 19F isolates (n = 68) obtained from children <5 years of age (*15*–*17*). New alleles were verified by resequencing gene fragments of both strands. When sequence types (STs) that had not been associated with a particular serotype in the past were identified, serotyping and sequencing typing were confirmed by repeating the reactions. STs were divided into sets using eBURST software (Imperial College, London, UK) (*18*), which is available at the MLST database. eBURST sets meet the requirement that all STs must be single-locus variants (SLVs) of at least 1 other ST within the group. Founder STs were defined as described previously (*18*). Clonal complexes (CCs) consisted of eBURST sets or eBURST sets plus related STs that shared 5 of 7 allelic identities with most other STs included within an eBURST set.

Antimicrobial Drug Susceptibility Testing and Detection of *erm*B/*mef*A Genes

MICs of serogroup 19 were determined for 6 antimicrobial drugs (penicillin, cefotaxime, chloramphenicol, tetracycline, clindamycin, and erythromycin) by E-test (AB Biodisk, Solna, Sweden) (*19*). Susceptibilities to vancomycin and trimethoprim-sulfamethoxazole were determined by disk diffusion test. Multidrug resistance was defined as nonsusceptibility to ≥3 antimicrobial drug classes. The *mef*A and *erm*B genes were detected by PCR using the primers *mef*A (forward, 5′-AGT ATC ATT AAT CAC TAG TGC-3′; reverse, 5′-TTC TTC TGG TAC TAA AAG TGG-3′) and *erm*B (forward, 5′-GAA AAG GTA CTC AAC CAA ATA-3′; reverse, 5′-GTA ACG GTA CTT AAA TTG TTT AC-3′) (*20*). PCRs were performed with 35 amplification cycles: 30 s at 94°C, 30 s at 50°C, and 1 min 30 s at 72°C, followed by a final extension at 72°C for 10 min.

Statistical Analysis

Statistical analysis was performed by using SPSS software version 13.0 (SPSS, Chicago, IL, USA). Serotype proportion in each period was compared using the χ^2 or Fisher exact test, as appropriate. The Mantel-Haenszel χ^2 test was used for trend analysis.

Results

Changes in Serotype Distributions

From 1991 through 2006, 538 strains of *S. pneumoniae* were obtained from various clinical specimens. Of these, 158 (29%) were from invasive isolates; 124 blood, 15 cerebrospinal fluid, 6 pleural fluid, 5 ascites, and 8 other sterile deep-seated tissues (e.g., bone and joint fluid). The remaining 380 (71%) were from noninvasive isolates; 110 (pharyngeal swab), 91 (transtracheal aspirate), 81 (sputum), 69 (middle ear fluid), 15 (urine), and 14 (open pus).

The most common serotypes were 19F (113, 21%), 23F (96, 17.8%), 19A (58, 10.8%), 6B (50, 9.3%), 6A (43, 8%), 14 (40, 7.4%), and 9V (24, 4.5%); these 7 serotypes accounted for 79% of the total isolates. Overall, PCV7 serotypes accounted for 64.1% of total isolates and 62.7% of invasive isolates. Table 1 shows the serotype distributions of invasive and noninvasive isolates by age group. For invasive isolates, PCV7 serotype coverage was 67% among children <60 months of age and 47% among children ≥60 months (Table 1). During 2001–2003, just before PCV7 was introduced in South Korea, overall PCV7 coverage rates for PCV7 serotypes and PCV7-related serotypes were 54% and 10% among the 138 invasive isolates and 57% and 13% among the 380 noninvasive isolates. The proportion of serotype 19A isolates increased from 0% (0/40) during 1991–1994 to 18% (7/39) during 2001–2003 among the 138 invasive isolates. Similarly, from 1995–1997 to 1998–2000, the proportion of serotype 19A isolates increased from 3% (1/33) to 17% (14/81) among noninvasive isolates (Figure 1).

Among the 107 invasive isolates from children <5 years of age (Figure 2), serotype 19F decreased from 31% (8/26) in period 1 to 13% (3/24) in period 2 and to 5% (1/20) in periods 3 and 4 (p = 0.008 for trend). The proportion of 19A increased from 0% (0/26) in period 1 to 8% (2/24) in period 2 and reached 26% (7/27) in period 4 (p = 0.005 for trend). There were no significant trends for the remaining serotypes: 23F (p = 0.58), 14 (p = 0.28), 9V (p = 0.23), 6A (p = 0.38), and 6B (p = 0.23). During period 5, after vaccine introduction, the proportion of 19A isolates was 20%

Table 1. Distributions of serotypes among 538 isolates from children in South Korea, by age group, 1991–2006*

Serotype	No. (%) invasive isolates†				No. (%) noninvasive isolates†			
	<24 mo	24–59 mo	≥60 mo	Total	<24 mo	24–59 mo	≥60 mo	Total
PCV7 serotypes	44 (67)	28 (68)	24 (47)	96 (61)	113 (65)	81 (71)	42 (45)	236 (62)
19F	8 (12)	6 (15)	4 (8)	18 (11)	50 (29)	33 (29)	12 (13)	95 (25)
23F	16 (24)	6 (15)	4 (8)	26 (16)	32 (18)	20 (18)	18 (19)	70 (18)
6B	8 (12)	3 (7)	7 (14)	18 (11)	19 (11)	10 (9)	3 (3)	32 (8)
14	7 (11)	10 (24)	5 (10)	22 (14)	8 (5)	7 (6)	3 (3)	18 (5)
9V	4 (6)	3 (7)	3 (6)	10 (6)	4 (2)	8 (7)	2 (2)	14 (4)
4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2)	4 (4)	6 (2)
18C	1 (2)	0 (0)	1 (2)	2 (1)	0 (0)	1 (1)	0 (0)	1 (0)
PCV7-related serotypes	3 (5)	6 (15)	7 (14)	16 (10)	15 (9)	14 (12)	9 (9)	38 (10)
6A	3 (5)	5 (12)	6 (12)	14 (9)	12 (7)	10 (9)	7 (7)	29 (8)
23A	0 (0)	1 (2)	1 (2)	2 (1)	3 (2)	4 (4)	0 (0)	7 (2)
9N	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2)	2 (1)
19A	11 (16)	2 (5)	0 (0)	13 (8)	27 (16)	8 (7)	10 (11)	45 (12)
Non-PCV7 serotypes	8 (12)	5 (12)	20 (39)	33 (21)‡	18 (11)	11 (10)	32 (35)	61 (16)
Total	66 (100)	41 (100)	51 (100)	158 (100)	173 (100)	114 (100)	93 (100)	380 (100)

*PVC7, 7-valent conjugate vaccine.
†Percentages have been rounded.
‡Thirteen serogroups were included among 33 invasive isolates as follows: 15 (6 strains), 24F (6), 10 (4), 34 (3), 35 (3), 1 (2), 3 (2), 12F (2), 5 (1), 11A (1), 13 (1), 20 (1), and 27 (1).

(2/10). During 2001–2003, 19A was the most common serotype among IPD isolates from South Korean children <5 years of age. During 2004–2006, the postvaccine period, when PCV7 uptake reached ≈20%–25% of South Korean children <2 years of age (*21*), the distribution of serotypes was unaltered compared with distribution during the prevaccine 2001–2003 period. There was no major change in antimicrobial drug treatment policy or pressure to use antimicrobial drugs in the local pediatric practice during the study period.

MLST Analysis of Serogroup 19

MLST analysis showed 4 STs among the 58 serotype 19A isolates: ST320 (52 isolates), ST 1374 (4), ST1451 (1), and ST2394 (1). Eighteen STs were found among the 68 serotype 19F isolates: ST271 (14 isolates), ST320 (6), ST236 (14), ST283 (7), ST1464 (10), ST2395 (3), ST2695 (3), and 1 isolate each of ST1203, ST1412, ST1417, ST2396, ST2397, ST2398, ST2399, ST2694, ST2696, ST2697, and ST2698. Through the course of this study, 11 new STs (ST2394-ST2399 and ST2694-ST2698) were identified among the serogroup 19 isolates, and 8 of these were SLVs or double-locus variants of ST271.

According to eBURST analysis, 1 major CC (CC271) accounted for most of the isolates (n = 116, 92% of serotypes 19A and 19F). CC271 comprised 16 STs, where ST271 was predicted as the founder, and ST320 was the predominant allelic profile. The 5 ST lineages that were unrelated to CC271 were nonlinked singlets (ST1203, ST1374, ST2394, ST2395, and ST2399) (Figure 3).

ST320 was the only ST found among serotypes 19A and 19F; ST320 was the most common ST (n = 52, 90% of total 19A isolates) among serotype 19A. ST320 was observed in only 9% (n = 6) of serotype 19F isolates. The genetic structure of serotype 19A comprised primarily ST1374 during periods 1 and 2 (1991–1997), but ST1374 isolates were not recovered after 2001. In contrast, ST320 was the most common sequence type from 1998, and all 19A isolates from 2002–2006 were of ST320. The numbers of 19A isolates increased consistently from 1996 through 2003, which suggests that single clonal expansion of ST320 was responsible for the increase of serotype 19A isolates during this period (Figure 4).

Unlike serotype 19A, serotype 19F comprises diverse STs grouped within CC271 (16 STs) and 5 different singlets. All STs, except ST1203, were associated with multidrug-resistant *mef/erm*-containing isolates. The distributions of predominant STs among serotype 19F isolates varied during each study period (Figure 4). For example, ST2395 predominated in period 1 (1991–1994), but ST1464 (an SLV of ST271) predominated in periods 4 and 5 (2001–2006).

Association of ST with Antimicrobial Drug Susceptibility and *mef*/*erm* Prevalence

Antimicrobial drug susceptibilities were tested for 126 of 171 serogroup 19 isolates. Degrees of resistance to penicillin and cefotaxime differed among isolates containing different STs. In addition, a distinct correlation was found between *mef/erm* prevalence and STs (Table 2). All CC271 isolates (n = 116, 16 different STs) were not susceptible to penicillin and cefotaxime and showed multidrug resistance to ≥3 antimicrobial drug classes. Erythromycin MICs of CC271 isolates were ≥256 μg/mL and were all positive for *mef* and *erm*. However, 5 singlets that were unrelated to CC271 showed different patterns of antimicrobial drug susceptibility and presence of *mef/erm* determinants (Table 2). Four serotype 19A isolates representing ST1374 appeared to be less resistant to 2 β-lactam antimicrobial drugs than

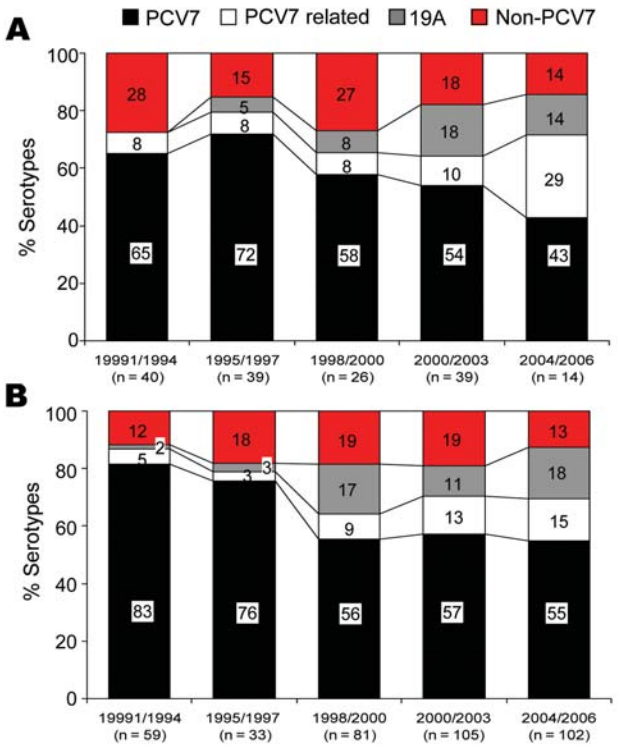

Figure 1. Distribution of serotypes with regard to 7-valent conjugate vaccine (PCV7) among 538 isolates encountered during five 3-year periods from 1991 through 2006, South Korea. A) Invasive isolates. B) Noninvasive isolates.

CC271 isolates. ST 1374 strains were also highly resistant to erythromycin (MIC ≥256 μg/mL) and were positive for *erm*B only. Strains of ST2394, ST2395, and ST2399 showed a lower degree of erythromycin resistance (MIC = 2–8 μg/mL) than CC271 and ST1374 and contained *mef*A only. One serotype, 19F strain of ST1203, did not express *mef* or *erm*. Serotypes 19A and 19F strains with CC271 were shown to be closely related to the internationally established clone, Taiwan^{19F}-14, which is multidrug resistant and carries *mef*A/*erm*B macrolide–resistance determinants defined by the pneumococcal molecular epidemiology network (*22*).

Discussion

We found that before PCV7 introduction in South Korea, the proportion of serotype 19A isolates increased from 0% in 1991 to 26% in 2003 but 19F isolates decreased during the same period. Our study also demonstrated that multidrug-resistant ST320 isolates containing *mef/erm* determinants were responsible for the expansion of serotype 19A.

In the United States, serotype 19A is now the most important cause of IPD by replacement serotypes (*4,8,23,24*). Contrary to what we report for South Korea, the increase in the United States was documented after widespread use of PCV7 (*4–6*). Increase in non-PCV7 serotypes, i.e., serotype replacement, has been noted in carriage studies and the prelicensure clinical trials (*11,25,26*). After widespread use of PCV7 in young children, replacement of serotypes for IPD has been described for several serotypes in previous studies (*4–6*). Of these, an increase in the incidence of IPD cases caused by serotype 19A was quite high. Therefore, the increasing prevalence of IPDs caused by serotype 19A among the vaccine target group is of considerable concern. Our findings of an increase in serotype 19A disease before conjugate vaccine introduction calls into question the role vaccine may play in the emergence of serotype 19A disease and suggests that other factors are important.

Of the factors contributing to the increase in serotype 19A, the MLST findings in our study point to a homogeneous pattern of ST320. ST320 of serotype 19A might have originated from ST271 or ST236 strains that have been prevalent among serotype 19F since 1993 (*20*) or could have been introduced from other countries. Thus, single clonal expansion of ST320, related to a multidrug-resistant internationally prevalent clone, Taiwan^{19F}-14 (that also carries *mef*A/*erm*B determinants), was most likely responsible for the prevaccine increase observed for serotype 19A. Antimicrobial drug use may provide selective pressure that would give this highly resistant strain an advantage over other strains. A study in the United States demonstrated an increasing prevalence of *mef*A/*erm*B; 17% percent of 221 children had been colonized by *mef*A/*erm*B containing serogroup 19 pneumococci strains after receiving at

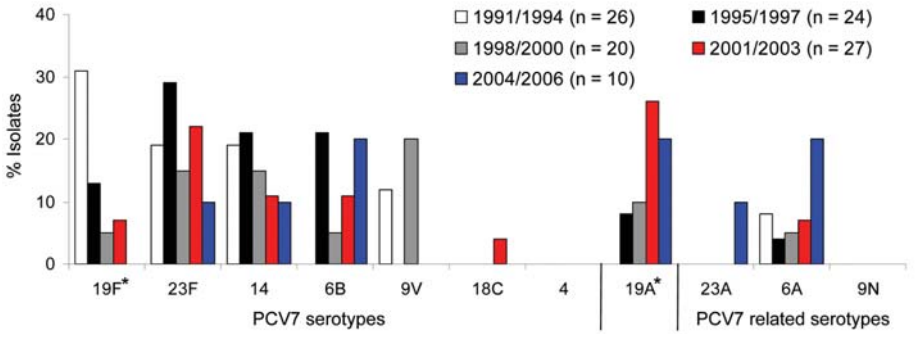

Figure 2. Distribution of serotypes with regard to 7-valent conjugate vaccine (PCV7) among 107 pneumococci isolated from children <5 years of age with an invasive pneumococcal infection (IPD) during five 3- or 4-year periods from 1991 through 2006, South Korea. *The observed increase in the proportion of 19A (p = 0.005) and decrease in the proportion of 19F (p = 0.008) among invasive isolates were statistically significant.

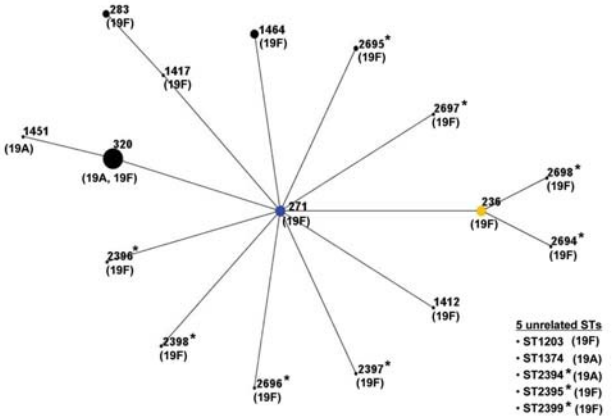

Figure 3. Relationship of 126 strains of serogroup 19 by eBURST analysis. ST271 (blue circle) is the predicted primary founder and ST236 (yellow circle) was assigned to a subgroup founder. Numbers on the diagram correspond to sequence types (STs). The size of each circle correlates with the number of isolates of that ST. *Newly identified ST in this study.

least 1 dose of PCV7; the major clonal type was related to Taiwan^{19F}-14 (*27*). In Alaska, the increase observed in 19A colonization and IPD seems to be related to CC172 clonal expansion (*8*). In contrast, recent genetic analysis of 19A strains isolated after PCV7 use in the United States showed that diverse mechanisms were involved in the expansion of 19A strains, expansion of preexisting predominant CC199, capsular switching of PCV7 types (4, 6, 14, and 9V), and appearance of multiple unrelated multidrug-resistant CCs among serotype 19A strains (CC271, including ST1451 and ST320, CC156, and CC1296) (*4*). Capsular switching of PCV7 serotypes under selective pressure by vaccine use is one of the mechanisms underlying the expansion of serotype 19A (*4,11–13,28*). However, there is no evidence of capsular switching as a contributing factor to the increase in serotype 19A in our study.

We also found that serotype 19F gradually decreased in proportion and diversified to include several newer descendants that differ from the founder by only 1 or 2 of 7 alleles but ST320 was not a major genetic structure among serotype 19F strains (*18*). Thus, it appears that ST320 has a selective advantage in serotype 19A strains, whereas ST320 did not seem to have expansion merit in the close serotype 19F strains. This finding suggests that the properties of particular clonal or capsular types are likely important determining factors of the potential of pneumococci to influence disease type and severity (*29,30*). Further studies are necessary to explain why certain sequence types exhibit different selective pressures according to serotype, even for close serotypes such as 19A and 19F.

This study has several limitations. First, pneumococcal isolates were collected at a single center, and thus, may not represent the national situation. However, no surveillance system has been established for IPD in South Korea. Nevertheless, at least 2 studies have demonstrated a recent increase in serotype 19A isolates among children in daycare centers (3% in 2002 and 11% in 2004) (*14,31*). In addition, the number of serotype 19A isolates from children and adults at another tertiary South Korean hospital showed an increase over the same period (*32*). Second, the number of 19A isolates was relatively small, and it is possible that uncommon strains possessing minor clones were not detected. Thus, our finding of a clonal expansion of ST320 among serotype 19A strains may be an overstatement. However, MLST analysis showed that all 7 of the 19A strains from colonized children who were identified from a previous study (*14*) were of ST320 (H.J. Lee, unpub. data).

The present study has implications for future pneumococcal immunization programs. In particular, the demonstration of an increase in 19A before the use of PCV7 suggests that PCV7 vaccination may not be entirely responsible for the observed increase of serotype 19A. Had the vaccine been introduced in 2000, as in the United States, the increase of serotype 19A would have been attributed to serotype replacement after PCV7 introduction. Nevertheless, whether minor multidrug-resistant clones of serotype 19A (appearing after the introduction of PCV7 in the United States) possess an advantage to increase with time is a concern (*23,29*).

We demonstrated that multidrug-resistant ST320 strains among serotype 19A have selective advantage in terms of expansion over ST1374, which were less resistant to β-lactams in a country where antimicrobial drug therapy is frequently used. Reports on PCV7 efficacy indicate negligible cross-protection for serotype 19A (*11*). In addi-

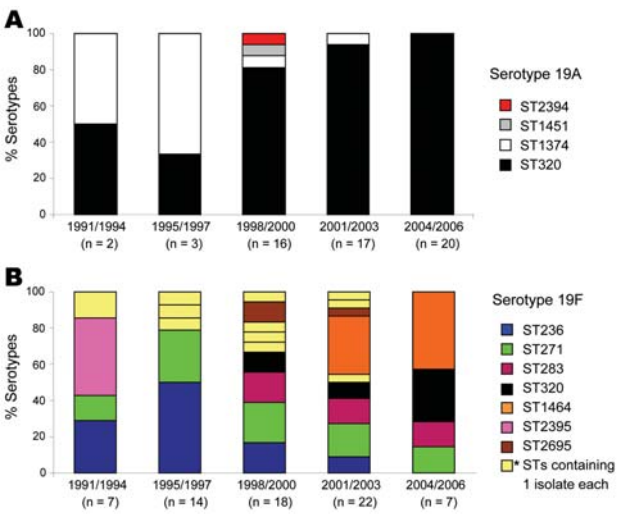

Figure 4. Distributions of sequence types (STs) of serotypes 19A and 19F during five 3-or 4-year periods from 1991 through 2006, South Korea. *Indicates 11 different STs that contained 1 isolate of serotype 19F. A) Invasive isolates. B) Noninvasive isolates.

Table 2. Antimicrobial susceptibility and *mef* and *erm* prevalence of serogroup 19 pneumococcal isolates from children in South Korea, 1991–2006, according to sequence type

CC or ST* (no. strains)	Serotypes	Multidrug resistance†	MIC_{50} (range) in μg/mL for each antimicrobial drug			*mef/erm* determinants
			Penicillin	Cefotaxime	Erythromycin	
CC271-related‡ (116)	19A, 19F	Yes	1.5 (1.0–3.0)	1.0 (0.75–4.0)	≥256 (≥256)	*mef+, erm+*
ST1203 (1)	19F	No	0.5	0.25	0.5	*mef–, erm–*
ST1374 (4)	19A	Yes	0.06 (0.04–0.06)	0.12 (0.09–0.12)	≥256 (≥256)	*mef–, erm+*
ST2394 (1)	19A	Yes	1.0	0.5	2.0	*mef+, erm*
ST2395 (3)	19F	Yes	4.0 (4.0)	2.0 (1.0–2.0)	2.0 (2.0)	*mef+, erm*
ST2399 (1)	19F	Yes	0.12	0.25	8.0	*mef+, erm*

*CC, clonal complex; ST, sequence type.
†Resistant to at least 3 antimicrobial drug classes.
‡CC271-related sequence types: ST320 (59 isolates), ST271 (14), ST236 (14), ST283 (7), ST1451 (1), ST1464 (10), ST2395 (3), ST2695 (3), and 1 isolate of each of ST1412, ST1417, ST2396, ST2397, ST2398, ST2694, ST2696, ST2697, and ST2698.

tion, a recent increase in the antimicrobial drug resistance of invasive 19A isolates and the increase in colonization by serogroup 19 strains carrying *mef*/*erm* determinants raise the possibility of potential increases in the prevalence of this clone. Thus, potential for colonization because of widespread antimicrobial drug use and resistance may interact and provide the selective advantage necessary for serotype expansion, which may be the situation in South Korea. Similarly, the influence of population characteristic dynamics, i.e., HIV infection or poverty and overcrowding, as well as PCV7 introduction, may combine with the necessary factor for serotype replacement and play an important role in serotype expansion, which may be the situation in Alaska. It is too early to determine the effect of PCV7 on expansion of serotype 19A in South Korea. PCV7 was introduced in South Korea in November 2003 when PCV7 serotype coverage was 56% among invasive isolates in children <5 years of age. In 2007, PCV7 coverage is ≈30% of the target group (*21*). At this time it is difficult to predict the effect of low vaccination coverage on serotype expansion/replacement. Therefore, surveillance is essential to monitor antimicrobial drug resistance, serotype expansion, and serotype replacement as early indications of an increase in pneumococcal disease by non-PCV7 or PCV7-related serotypes.

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Molecular Typing of Australian *Scedosporium* Isolates Showing Genetic Variability and Numerous *S. aurantiacum*

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One hundred clinical isolates from a prospective nationwide study of scedosporiosis in Australia (2003–2005) and 46 additional isolates were genotyped by internal transcribed spacer–restriction fragment length polymorphism (ITS-RFLP) analysis, ITS sequencing, and M13 PCR fingerprinting. ITS-RFLP and PCR fingerprinting identified 3 distinct genetic groups. The first group corresponded to *Scedosporium prolificans* (n = 83), and the other 2 comprised isolates previously identified as *S. apiospermum*: one of these corresponded to *S. apiospermum* (n = 33) and the other to the newly described species *S. aurantiacum* (n = 30). Intraspecies variation was highest for *S. apiospermum* (58%), followed by *S. prolificans* (45%) and *S. aurantiacum* (28%) as determined by PCR fingerprinting. ITS sequence variation of 2.2% was observed among *S. apiospermum* isolates. No correlation was found between genotype of strains and their geographic origin, body site from which they were cultured, or colonization versus invasive disease. Twelve *S. prolificans* isolates from 2 suspected case clusters were examined by amplified fragment length polymorphism analysis. No specific clusters were confirmed.

Despite efforts to identify and eliminate infectious agents, they continue to emerge and reemerge (*1*). Among them, pathogenic fungi contribute substantially to illness and death, especially in immunocompromised patients (*2,3*). In contrast to the well-documented opportunists *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, the epidemiology and evolution of human infections caused by uncommon but emerging fungi are incompletely understood. Such pathogens include *Scedosporium apiospermum* (teleomorph *Pseudallescheria boydii*) and *S. prolificans,* which are inherently resistant to many antifungal agents (*3–5*).

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S. apiospermum infections occur worldwide, ranging from localized mycetomas to deep-seated disease such as cerebral abscesses (*6,7*). This species also colonizes the respiratory tract of ≈10% of patients with cystic fibrosis and chronic suppurative lung disease (*8–10*). On the basis of genetic data, a new species, *S. aurantiacum,* was proposed for a subset of isolates previously identified as *S. apiospermum* (*11*). *S. prolificans* infections are geographically more restricted than those caused by *S. apiospermum,* being most prevalent in Australia, Spain, and the United States (*12–15*). *S. prolificans* typically causes localized infections in immunocompetent hosts but rapidly fatal disseminated infections in the immunocompromised among whom it has been associated with nosocomial outbreaks (*3*,*12–17*).

Since scedosporiosis, in particular that caused by *S. prolificans*, is often refractory to treatment (*3*,*5*), preventive strategies are of paramount importance. However, the epidemiology and mode of transmission of infection are not well understood. Furthermore, the environmental reservoir of *S. prolificans* is unknown. Molecular typing techniques now provide the means to elucidate the epidemiology of *Scedosporium* infections and to investigate potential case clusters (*16*,*18,19*). Strains recovered from patients with cystic fibrosis have demonstrated a high degree of genetic variability (*10*,*20*), although a single genetic profile predominated in 1 study (*8*). The degree of genetic variation within *S. prolificans* is more controversial. Two studies have reported low to no intraspecies genetic heterogeneity (*16*,*21*), while a third noted substantial genetic diversity (*19*). The results of these studies may be biased because they included only small numbers of isolates from specific patient populations. Genetic variability among *S. aurantiacum* has not yet been studied.

In this study, we used 4 molecular tools to examine genetic variation among a large number of Australian clinical *Scedosporium* isolates: 1) internal transcribed spacer (ITS)–based restriction fragment length polymorphism (ITS-RFLP) analysis; 2) DNA sequence analysis of the ITS region (selected isolates); 3) PCR fingerprinting using the microsatellite specific core sequence of phage M13; and 4) amplified fragment length polymorphism (AFLP) analysis (isolates from suspected case clusters). We also searched for the newly described species, *S. aurantiacum* and for genetic clustering of strains according to their geographic origin, body site from which they were cultured, and ability to cause invasive disease.

Materials and Methods

Scedosporium Isolates and Data Collection

A total of 146 *Scedosporium* isolates were studied (online Technical Appendix, available from www.cdc.gov/EID/content/14/2/282-Techapp.pdf). Forty-six were from the culture collection at the Clinical Mycology Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services, Westmead Hospital, Sydney, Australia. For these isolates, the following data were captured: demographic information, patient coexisting conditions and risk factors (summarized in the online Technical Appendix). The remaining 100 isolates were obtained through a national, prospective, laboratory-based surveillance for scedosporiosis in Australia (the Australian *Scedosporium* [AUSCEDO] Study) from January 2003 to December 2005. The following data were collected: clinical status, risk factor (defined according to published risk factors for scedosporiosis [*4,12–15*]), major comorbidity (based on the International Classification of Diseases, 10th revision, Australian Modification [ICD-10 AM] diagnostic classification system [*22*]), isolated species, treatment and outcome. *Scedosporium* strains obtained from a single colony from the primary isolation plate from all patients were forwarded to the Molecular Mycology Research Laboratory, Westmead Hospital, for genotyping. Isolates were identified as *S. prolificans* or *S. apiospermum* by standard phenotypic methods (*23*). Species were confirmed as *S. prolificans* or *S. apiospermum*, and *S. aurantiacum* was identified (*11*) by ITS-RFLP analysis.

Definitions

An episode of scedosporiosis was defined as the incident isolation of *Scedosporium* spp. from any body site. Two or more episodes, fulfilling the case definition and occurring in different patients that were epidemiologically linked were defined as a potential case cluster. Invasive disease was defined according to the European Organization for Treatment of Cancer/Mycoses Study Group criteria for "definite" or "probable" infection (*24*). All other patients not fulfilling these criteria, including those with "possible" infection were considered colonized. Coincident hospital renovations or construction was considered to be a potential risk factor if major work was undertaken within 3 months before the isolation of *Scedosporium* spp. from a patient.

Description of 2 Potential Case Clusters

The first potential case cluster involved 8 patients located in the same hematology/hemopoietic stem cell transplant (HSCT) unit at the Alfred Hospital, a large university hospital in Melbourne (September 2000–October 2001; [*15*]). The second consisted of 3 patients located in the same hematology/HSCT ward at Westmead Hospital a major university hospital in Sydney (September 2003–January 2004; unpub. data). Details of the patients involved in these suspected case clusters are summarized in the online Technical Appendix). On each occasion, patient isolates were submitted for genetic analyses to inform infection control responses (see Results).

Genomic DNA Extraction and ITS-RFLP Analysis

Genomic DNA was isolated as described previously (*18*). The ITS1, 5.8S, and ITS2 regions of the rDNA gene cluster were amplified with the primers SR6R and LR1 (Table 1) as described previously (*25*). Amplicons were double digested with the restriction endonucleases *Sau*96I and *Hha*I (New England BioLabs, Ipswich, MA, USA) in accordance with the manufacturer's recommendations. Digested products were separated by electrophoresis in 3% agarose gels at 100 V for 3–4 h. Banding patterns were analyzed visually.

ITS Sequencing

Eleven isolates, representative of each of 3 ITS-RFLP patterns obtained, were selected for ITS sequencing: ITS-RFLP profile A (*S. prolificans*, WM 06.378, WM 06.440, and WM 06.393), ITS-RFLP profile B (*S. apiospermum*, WM 06.389, WM 06.471, and WM 06.497), and ITS-RFLP profile C (*S. aurantiacum,* WM 06.388, WM 06.482, WM 06.495, WM 06.496, and WM 06.498). The ITS region was amplified as described above and commercially sequenced in both directions by using SR6R or LR1 (Table 1) as forward and reverse primers.

PCR Fingerprinting

The minisatellite-specific core sequence of the wild-type phage M13 was used as a single primer for PCR fingerprinting (Table 1). Amplification reactions were performed as previously described (*18*). Blank control tubes containing all reagents except template DNA were included for each run; each sample was analyzed at least twice. PCR products were separated by electrophoresis on 1.4% agarose gels at 60 V for 14 cm. Strains were defined to be identical if their PCR fingerprinting profiles had a similarity of ≥97% (= 1 band difference). Reproducibility of the PCR fingerprinting technique was accessed by re-amplifying 1 strain of each of the 3 *Scedosporium* spp. with all PCR amplifications carried out and re-running those on each gel.

AFLP Analysis

AFLP analysis was performed as described previously by using either *Eco*RI-GT 6-FAM-labeled and *Mse*I-GT or *Eco*RI-TC 6-FAM-labeled and *Mse*I-CA as selective primer pairs (QIAGEN, Valencia, CA, USA; Table 1) (*26*). All samples were analyzed by using the ABI Prism 3730 system (Applied Biosystems, Foster City, CA, USA). Data collation, fragment sizing, and pattern analyses were performed with GeneMapper software version 3.5 (Applied Biosystems). Only electrophoregram peaks above 1,000 fluorescent units were scored for the presence or absence of bands of the same size (range 50–500 bp) relative to the GeneScan 500 LIZ DNA size standard (Applied Biosystems). Only bands detected in duplicate AFLP experiments were included in the analysis.

Data Analysis

Clinical Data

Statistical analysis was performed by using SPSS version 10.0.07 (SPSS, Chicago, IL, USA) and EpiInfo version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Proportions were compared by using the χ^2 or Fisher exact test. A p value <0.05 was statistically significant.

ITS Sequences

ITS sequences obtained from 11 isolates (see above) were aligned with the ITS sequences of the following reference strains obtained from GenBank: *S. apiospermum* CBS 101.22 (accession no. AJ888435), *S. aurantiacum* FMR 8630 (accession no. AJ888440), *S. aurantiacum* IHEM 15458 (accession no. AJ888441) and *S. prolificans* CBS 114.90 (accession no. AY882369) as well as 2 outgroup sequences: *Pseudallescheria africana* CBS 311.72 (accession no. AJ888425), and *Petriella setifera* CBS 164.74 (accession no. AY882352). Phylogenetic analyses were performed by using PAUP* version 4.06.10 (*27*).

PCR Fingerprinting Patterns and AFLP Fragments

PCR fingerprinting patterns were analyzed by using the 1D gel analysis module (BioGalaxy [BioAware, Hannut, Belgium]) in BioloMICS version 7.5.30 (BioAware). Images were normalized for lane to-lane differences in mobility by the alignment of patterns obtained on multiple loadings of the 1kb DNA size marker (GIBCO-BRL, Gaithersburg, MD, USA). The unweighted-pair group method by using arithmetic averages and the procedures of Nei and Li (*28*), both implemented in BioloMICS, were used to generate dendograms based on the coefficient of similarity (*29*) between the isolates. In addition, principal coordinate analysis (PcoA; BioloMICS) was conducted to give an overall representation of the observed strain variation. AFLP fragments were analyzed with BioloMICS.

Results

A total of 146 *Scedosporium* isolates from 120 episodes (119 patients) were studied (online Technical Appendix). Demographic data were available for 108 (90%) episodes and coexisting conditions and risk factor data for 115 (95.8%). Most episodes were reported from New South Wales (64.2%), followed by Victoria (19.2%) and Western Australia (9.2%). The male: female ratio was 1.3: 1. The major patient coexisting conditions and known risk factors for scedosporiosis are summarized in the online Technical Appendix. Thirty-nine patients (32.7%) had no underlying medical condition. Coincident building construction was noted in 27 cases (22.5%). *Scedosporium* isolates were associated with invasive disease in 46 (38.3%) instances;

Table 1. Primer and adaptor oligonucleotide sequences used in the study

Primer or adaptor oligonucelotide	Sequence (5′→3′)
rDNA primers	
SR6R	AAGTARAAGTCGTAACAAGG
LR1	GGTTGGTTTCTTTTCCT
M13 fingerprinting primer	
Phage M13	GAGGGTGGCGGTTCT
*Eco*RI adapters	
EA1	CTCGTAGACTGCGTACC
EA2	CATCTGACGCATGGTTAA
*Mse*I adapters	
MA1	GACGATGAGTCCTGAG
MA2	TACTCAGGACTCAT
Preselective primers	
*Eco*RI-T	GACTGCGTACCAATTCT
*Eco*RI-G	GACTGCGTACCAATTCG
*Mse*I-C	GATGAGTCCTGAGTAAC
*Mse*I-G	GATGAGTCCTGAGTAAG
Selective primers	
*Eco*RI-TG	6 FAM-GACTGCGTACCAATTCTG
*Eco*RI-GT	6 FAM-GACTGCGTACCAATTCGT
*Mse*I-CA	GATGAGTCCTGAGTAACA
*Mse*I-GT	GATGAGTCCTGAGTAAGT

the remaining 74 (61.7%) were isolated from patients who were colonized (Table 2).

Molecular Typing of *Scedosporium* Isolates

All 146 isolates were examined by ITS-RFLP analysis and PCR fingerprinting. ITS sequencing was performed on 11 strains as described above. AFLP analysis was performed only for selected *S. prolificans* isolates, including the isolates of the suspected case clusters and isolates representative of the *S. prolificans* branches identified by PCR fingerprinting (online Appendix Figure 1, available from www.cdc.gov/EID/content/14/2/282-appG1.htm).

ITS-RFLP Analysis

RFLP analysis found 1 RFLP profile specific for *S. prolificans* isolates (ITS-RFLP profile A) and 2 profiles (ITS-RFLP profiles B and C) for isolates previously phenotypically identified as *S. apiospermum* (Figure 1, panel A). ITS-RFLP profile B corresponded to *S. apiospermum* and ITS-RFLP profile C to the newly described species, *S. aurantiacum*.

ITS Sequencing

Sequencing of the ITS 1, 5.8S, and ITS2 regions of the 11 strains, representative of each of the 3 ITS-RFLP profiles found the following results: BLAST searches against the corresponding GenBank reference sequences identified strains: WM 06.389 (accession no. EF639870), WM 06.497 (accession no. EF639872), and WM 06.471 (accession no. EF639871) (ITS-RFLP profile B) as *S. apiospermum* (96%–99% sequence similarity to strain CBS 101.22). Strains WM 06.388 (accession no. EF639865), WM 06.482 (accession no. EF639866), WM 06.495 (accession no. EF639867), WM 06.496 (accession no. EF639868), and WM 06.498 (accession no. EF639869) (ITS-RFLP profile C) were identified as *S. aurantiacum* (100% sequence identity with strains FMR 8630 and IHEM 15458). Isolates WM 06.393 (accession no. EF639863), WM 06.440 (accession no. EF639864) and WM 06.378 (accession no. EF639862) (ITS-RFLP profile A) were identified as *S. prolificans* (100% identity with strain CBS 114.90).

Phylogenetic analysis of the sequences demonstrated 3 distinct clades, the first corresponding to *S. prolificans* as the basal clade. The other 2 corresponded to the 2 more closely related but clearly distinct clades, *S. apiospermum,* and *S. aurantiacum* (Figure 2). *S. apiospermum* showed intraspecies sequence variation of 2.2% compared to *S. aurantiacum* and *S. prolificans,* which displayed no variation.

Final Identification of *Scedosporium* spp. and Clinical Associations

S. prolificans accounted for 75 patient episodes (83 of 146 isolates; 56.9%), *S. apiospermum* for 25 (33 isolates; 22.6%), and *S. aurantiacum* for 23 (30 isolates; 20.6%) (online Technical Appendix). More than 1 *Scedosporium* spp. was isolated from the same patient in 3 instances: Patient 83: *S. apiospermum* (WM 06.471, WM 06.472, WM 06.474, and WM 06.475) and *S. prolificans* (WM 06.473); patient 91: *S. apiospermum* (WM 06.486) and *S. prolificans* (WM 06.485); and patient 102: *S. apiospermum* (WM 06.500) and *S. prolificans* (WM 06.501) (online Technical Appendix). In 6 episodes, the same species was recovered from more than 1 body site in the same patient at the same time (patients 57 [blood, bronchial washing, skin], 73 [blood, sputum], 80 [sputum, bone, wound fluid], 83 [bronchial washing, bronchoalveolar lavage], 118 [pleural fluid, bone, wound fluid, chest tissue], and 119 [blood, skin]; online Technical Appendix).

Approximately half (40%–52.2%) of *S. apiospermum* and *S. aurantiacum* isolates were from the respiratory tract/lung compared to 20% for *S. prolificans*. Conversely, all isolates from blood, 57.2% isolates from skin/soft tissue and 66.7% from eye were *S. prolificans* (Table 2). Invasive disease was more likely to be caused by *S. prolificans* than non-*prolificans Scedosporium* spp. (83% versus 17% of isolations; odds ratio (OR) 5.3, 95% confidence interval (CI) 2.0, 14.2, $p = 0.002$) (Table 2). This association was significant when compared with *S. apiospermum* as well as with *S. aurantiacum* ($p<0.05$; data not shown). The relative proportions of invasive disease among *S. apiospermum* and *S. aurantiacum* were similar (Table 2). Coincident building construction (27 cases, 22.5%) was more likely to be

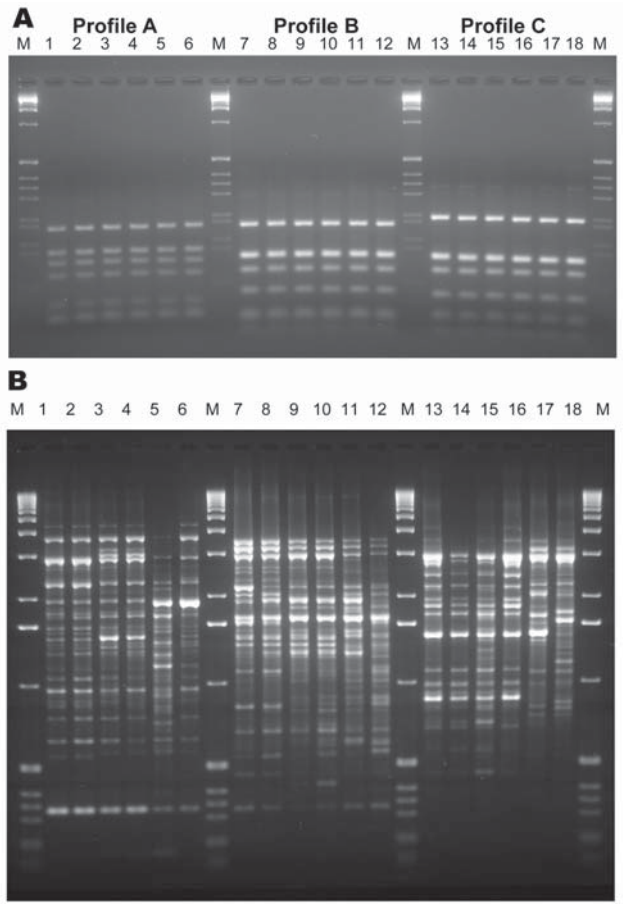

Figure 1. Internal transcribed spacer–restriction fragment length polymorphism (ITS-RFLP) patterns obtained by double digestion with the enzymes *Sau*96I and *Hha*I (A) and of the PCR fingerprinting profiles obtained with the microsatellite specific primer M13 (B) for *Scedosporium prolificans*: lane 1, WM 06.457; lane 2, WM 06.458; lane 3, WM 06.503; lane 4, WM 06.502; lane 5, WM 06.399; lane 6, WM 06.434. *S. aurantiacum*: lane 7, WM 06.495; lane 8, WM 06.496; lane 9, WM 06.386; lane 10, WM 06.385; lane 11, WM 06.482; lane 12, WM 06.390. *S. apiospermum*: lane 13, WM 06.475; lane 14, WM 06.474; lane 15, WM 06.472; lane 16, WM 06.471; lane 17, WM 06.424; lane 18, WM 06.443; lane M, 1-kb marker (GIBCO-BRL, Gaithersburg, MD, USA).

associated with isolation of *S. prolificans* compared with non-*prolificans Scedosporium* spp. (OR 11.5, 95% CI 2.4, 74.5; p<0.001; data not shown).

Molecular Epidemiology

Strain Typing

PCR fingerprinting delineated 3 major clusters concordant with *S. apiospermum*, *S. aurantiacum,* and *S. prolificans* (online Appendix Figure 1; Figure 1, panel B; Figure 3). Clusters corresponding to *S. aurantiacum* and *S. prolificans* were substantially more densely grouped than the *S. apiospermum* cluster (Figure 3).

PCR fingerprinting profiles showed polymorphisms within each of the 3 species, allowing for a clear differentiation, by using a "cut-off point" of ≥97% similarity. Multiple isolates from the same patient obtained from different anatomic sites (online Technical Appendix) had identical or ≥97% similarity between their PCR fingerprints, except for 1 patient (patient 118). In 8 instances, PCR fingerprinting showed that patients were infected with 2 different strains: (patients 1, 10, 27, 57, 83 99, 118 [online Appendix Figure 1, online Technical Appendix]). For all species, genetic profiles were independent of geographic origin, body site of isolation or whether the patient was infected or colonized (online Appendix Figure 1). Profiles were also independent of patient comorbidityity and risk factors for scedosporiosis (data not shown). Intraspecies PCR fingerprinting variation was highest for *S. apiospermum* (58%) followed by *S. prolificans* (45%) and *S. aurantiacum* (28%) (online Appendix Figure 1).

Examination of Isolates from Suspected Case Clusters

Twelve isolates from 2 presumptive case clusters of *S. prolificans* infection (Alfred Hospital, Melbourne pa-

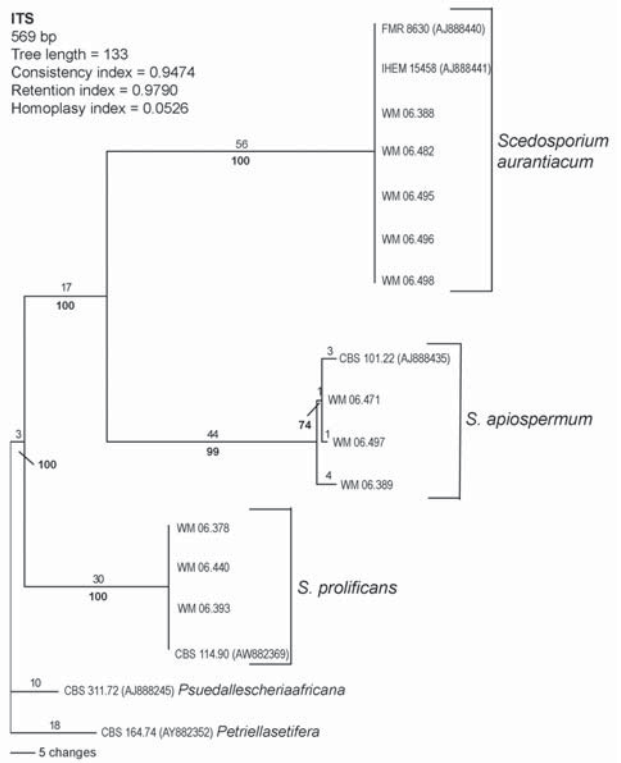

Figure 2. Rooted phylogram (outgroup *Pseudallescheria africana* CBS 311.72 and *Petriella setifera* CBS 164.74), showing the relationships among 11 selected strains representing each obtained internal transcribed spacer (ITS)–restriction fragment length polymorphism pattern and 4 reference strain sequences obtained from GenBank by using PAUP* version 4.06.10 (*29*).

Table 2. Selected characteristics for 120 isolations (episodes) of *Scedosporium* spp.

Characteristic*	No. *Scedosporium prolificans;* n = 75 (%)†	No. *S. apiospermum;* n = 25 (%)†	No. *S. aurantiacum;* n = 23 (%)†
Male sex	40 (53.3)	12 (48)	8 (34.8)
Risk factor			
Surgery ≤30 d	3 (4)	1 (4)	–
Trauma	5 (6.7)	–	1 (4.4)
Clinical status‡			
Invasive disease	39 (52)	4 (16)	4 (17.4)
Colonization	36 (48)	21 (84)	19 (82.6)
Body site of isolation			
Blood	18 (24)	–	–
Eye	2 (2.7)	–	1 (4.4)
Skin/soft tissue	4 (5.3)	1 (4)	2 (8.7)
Lung/respiratory tract	15 (20)	10 (40)	12 (52.2)
Ear	4 (5.3)	10	5 (21.2)

*Some patients had *Scedosporium* isolated from more than 1 body site.
†Refers to no. episodes in which each species was isolated. The total no. of isolates was 146 comprising 83 *S. prolificans*, 33 *S. apiospermum,* and 30 *S. aurantiacum*.
‡More than 1 *Scedosporium* spp. was isolated from 4 patients.

tients: isolates WM 06.392, WM 06.393, WM 06.395, WM 06.399, WM 06.400, WM 06.401, WM 06.402, and WM 06.405; Westmead Hospital, Sydney patients: isolates WM 06.432, WM 06.434, WM06.457, and WM 06.458; online Technical Appendix) as well as 23 additional isolates, representative of the *S. prolificans* branches identified by PCR fingerprinting (online Appendix Figure 1) were further investigated by AFLP typing. *S. prolificans* was not isolated from the environment in either setting despite extensive sampling. The AFLP bands were found to be 50–493 bp by using the primers *Eco*RI-GT and *Mse*I-GT (data not shown), and from 52–468 bp by using the primers *Eco*RI-TG and *Mse*I-CA (online Appendix Figure 2, available from www.cdc.gov/EID/content/14/2/282-appG2.htm). These 35 isolates exhibited 32 different AFLP profiles, with isolates from the same patient (patients 1, 73, and 119) showing identical profiles (online Appendix Figure 2), confirming the PCR fingerprinting results (online Appendix Figure 1). PcoA of the combined AFLP and PCR fingerprinting data demonstrated no clustering of these isolates (Figure 4), which ruled out the possibility of nosocomial transmission.

Discussion

We examined genetic variation among a large number of population-derived *Scedosporium* isolates across the Australian continent. In line with previously reported genetic variability in the *S. apiospermum/P. boydii* species complex (*30–32*), we observed 2 distinct ITS-RFLP patterns among *S. apiospermum* isolates, showing the presence of the newly described species *S. aurantiacum* (*11*). Notably, we have identified by ITS sequencing that *S. aurantiacum* comprised 45% of the current collection of Australian "*S. apiospermum"* isolates and documents genetic variability within *S. aurantiacum*.

Epidemiologic investigation of *Scedosporium* infection requires accurate identification and typing. *S. apiospermum*, *S. aurantiacum,* and *S. prolificans* were clearly distinguished from each other by PCR fingerprinting and ITS-RFLP analysis. This is consistent with previous rDNA sequence-based studies (*30*,*33*,*34*). The observation of 2 distinct genetic groups, corresponding to *S. aurantiacum* and *S. apiospermum,* supports the proposal that *S. aurantiacum* be designated a separate species (*11*). This proposal is also supported by the 5%–10% ITS sequence variation found between *S. aurantiacum* and *S. apiospermum* compared to an absence of intraspecies variation in *S. aurantiacum* and *S. prolificans* and a 2.2% variation in *S. apiospermum* (*30,32;* current study).

Using PCR fingerprinting, intraspecies variation was greatest (58%) among *S. apiospermum* isolates (Figure 3). This diversity is generally consistent with the high degree of polymorphism (15–20 genotypes) previously found (*10,20,32*). In contrast, genetic variation was lowest (28%) among the *S. aurantiacum* isolates (online Appendix Figure 1; Figure 3). Nevertheless, PCR fingerprinting polymorphisms clearly differentiated all 30 strains (online Appendix Figure 1). Further genotyping studies of a greater number of and more geographically diverse *S. aurantiacum* isolates are warranted.

The intraspecies PCR fingerprint variation in *S. prolificans* (45%) was greater than that in *S. aurantiacum* but less than that in *S. apiospermum.* Given that *S. aurantiacum* is phylogenetically more closely related to *S. apiospermum* than to *S. prolificans* (*11,33;* current study), this result was unexpected. It may be due to different evolutionary pressures acting on the 3 different species or the relatively small numbers of *S. aurantiacum* isolates studied to date. The moderate genetic diversity among *S. prolificans* confirms previous findings (*19*). Despite the observed

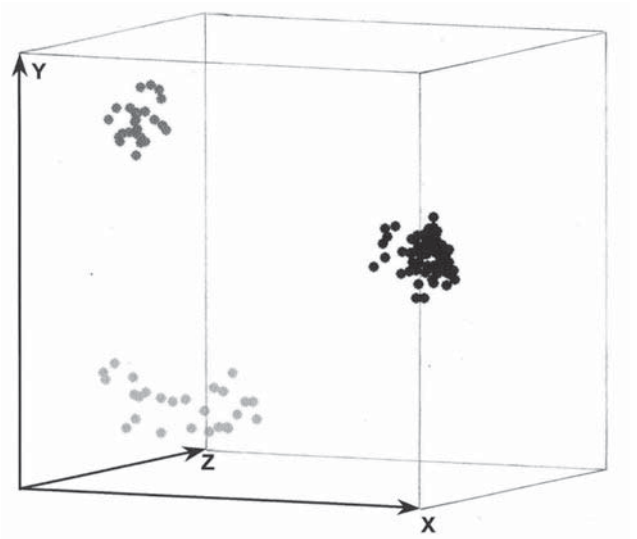

Figure 3. Three-dimensional presentation of the principal coordinate analysis of the PCR fingerprinting data showing 3 distinct clusters which correspond to *Scedosporium prolificans* (black dots), *S. aurantiacum* (dark gray dots), and *S. apiospermum* (light gray dots), with *S. apiospermum* showing the highest genetic variation.

polymorphisms, PcoA of PCR fingerprint profiles showed dense clustering for *S. prolificans* (Figure 3), which is consistent with the low to absent intraspecies variability in *S. prolificans* found by others (*20*,*21*,*33*). These apparently contradictory findings emphasize the importance of choosing the optimum molecular typing tool with the most appropriate discriminatory power for the organism or species being studied.

The high degree of intraspecies variation detected by PCR fingerprinting and AFLP analysis supports the use of these methods to establish genetic relatedness between isolates recovered from different patients or multiple isolates from the same patient. In comparison, the variation detected by ITS-RFLP analysis and ITS sequencing corresponded to interspecies variation, which makes those techniques ideal for identification of any given isolate to the species level. Individual patients are most likely infected or colonized with genetically distinct strains (*19–21;* this study). Identical PCR fingerprint or AFLP profiles were noted in multiple isolates recovered simultaneously from different anatomic sites in the same patient (*21*; current study). However, 8 patients were infected or colonized by at least 2 strains as reflected by their different genetic profiles (online Technical Appendix). Possible explanations include concomitant infection by multiple strains from which only a restricted number were recovered, or colonization by 1 strain followed by infection or colonization with a second strain of a different genotype. Longitudinal genotyping studies are required to determine the likelihood that persistence of ≥1 genotypes later leads to clinically important infection or whether the disease is more likely to be caused by an unrelated genotype. In this context, the development of a multilocus sequence typing scheme for *Scedosporium,* as has been developed for *Candida* spp. (*35*), would be of great advantage to overcome interlaboratory reproducibility problems, which are known to be associated with PCR fingerprinting or AFLP data. However, developing such a scheme remains cumbersome due to the current lack of genomic data of *Scedosporium* spp.

For all 3 *Scedosporium* spp., there was no clustering of strains according to their geographic or body site of origin or by their ability to cause invasive disease, which is in agreement with previous findings for *S. apiospermum* (*20*,*30*) and *S. prolificans* (*16*,*17*,*21*). Of note, no specific genotypes were associated with underlying medical conditions or risk factors. Compared with *S. apiospermum* and *S. aurantiacum S. prolificans* was more frequently associated with coincident hospital renovation, and invasive disease, had a greater predilection to cause disseminated infection and was the predominant species isolated from blood and other sterile sites (*12–16,36*; current study). Our preliminary observations indicate that the epidemiology and clinical relevance of recovering *S. aurantiacum* may be similar to that of *S. apiospermum. S. aurantiacum* has been reported to colonize the respiratory tract of at-risk patients (*8*).

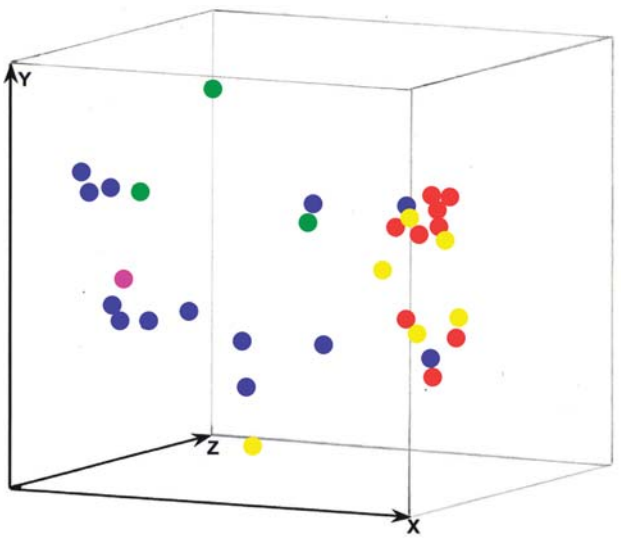

Figure 4. Three-dimensional presentation of the principal coordinate analysis of the combined M13 PCR fingerprinting, amplified fragment length polymorphism (AFLP) primers EcoRI-GT and MseI-GT, and AFLP primers EcoRI-TG and MseI-CA data from the suspected Sydney and Melbourne case cluster isolates and 23 other Australian isolates. None of the investigated isolates showed any epidemiologic connection except 3 isolates obtained from the same patient (nos. 1, 73, 119). Blue dots, Melbourne outbreak isolates; pink dot, Melbourne-related isolate; red dots, Sydney outbreak isolates; green dots, Sydney-related isolates; yellow dots, unrelated Australian isolates.

In addition to PCR fingerprinting, we applied AFLP analysis to investigate the possibility of 2 case clusters caused by *S. prolificans*. AFLP analysis was chosen as an independent technique using 2 combinations of selective primers (Table 1), which have been previously shown to have good discriminatory power for fungal strain differentiation (*26*). Both techniques, previously used to identify outbreak strain clusters in the recent cryptococcosis outbreak on Vancouver Island (*37*), generated in the current situation distinct patterns from all *S. prolificans* isolates except serial isolates obtained from the same patient (online Appendix Figures 1, 2). These findings exclude the occurrence of nosocomial outbreaks or any close relationship with the nonoutbreak isolates, a result similar to those obtained previously (*38*). Overall nosocomial acquisition of infection has been demonstrated in only 2 instances (*16,17*). *Scedosporium* spp. have rarely been isolated from hospital air or from indoor or outdoor surface samples (*13*,*39*,*40,* current study), which raises questions about the mode of acquisition by patients and the mechanisms of the selection of this specific fungus as an infectious agent from among the high biodiversity of environmental molds.

In conclusion, ITS-RFLP analysis is a powerful tool for distinguishing between isolates of the new species *S. aurantiacum* and *S. apiospermum*. PCR fingerprinting and AFLP analysis are useful techniques for determining genetic relatedness between *Scedosporium* isolates and for investigating potential case clusters.

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Emergence of Polycystic Neotropical Echinococcosis

Dennis Tappe,*† August Stich,† and Matthias Frosch*

Echinococcosis is a parasitic zoonosis of increasing concern. In 1903, the first cases of human polycystic echinococcosis, a disease resembling alveolar echinococcosis, emerged in Argentina. One of the parasites responsible, *Echinococcus oligarthrus*, had been discovered in its adult strobilar stage before 1850. However, >100 years passed from the first description of the adult parasite to the recognition that this species is responsible for some cases of human neotropical polycystic echinococcosis and the elucidation of the parasite's life cycle. A second South American species, *E. vogeli*, was described in 1972. Obtaining recognition of the 2 species and establishing their connection to human disease were complicated because the life cycle of tapeworms is complex and comprises different developmental stages in diverse host species. To date, at least 106 human cases have been reported from 12 South and Central American countries.

Echinococcosis is a parasitic zoonosis characterized by the development of a larval tapeworm stage (metacestode) in herbivorous intermediate hosts, such as rodents and ungulates, and accidentally in humans. The adult tapeworm is minute and inhabits the small intestine of canids or felids, the definitive hosts. Infections occur in intermediate hosts when they ingest eggs that have been passed in the feces of definitive hosts. In the past, many *Echinococcus* species have been described, but most have been abandoned or reclassified. Molecular phylogeny reconstructions are complex, and the process of taxonomic revision has not yet been completed (*1*). The causative agent of cystic echinococcosis (hydatidosis), the dog tapeworm *E. granulosus* sensu lato, is cosmopolitan. The species responsible for alveolar echinococcosis (AE), the fox tapeworm *E. multilocularis*, is endemic to Holoarctic regions. Recently, *E. shiquicus* n. sp. was discovered in Tibet (*2*). The "neotropical" echinococcal species *E. oligarthrus* and *E. vogeli* are confined to the New World. Either species is capable of causing polycystic echinococcosis (PE) in its natural intermediate host and accidentally in humans. Disease due to *E. vogeli* is similar to AE and is characterized by aggressive infiltrative growth and external budding, whereas infection with *E. oligarthrus* has a more benign course. PE thus comprises 2 disease entities. Each is characterized by distinctive epidemiology, clinical manifestations, and morphologic features of the adult and larval parasite (*3*). Today, PE is no longer a medical rarity as more and more cases are being discovered. The prevalence of the disease, however, is unknown.

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First Description of Human Neotropical Echinococcosis

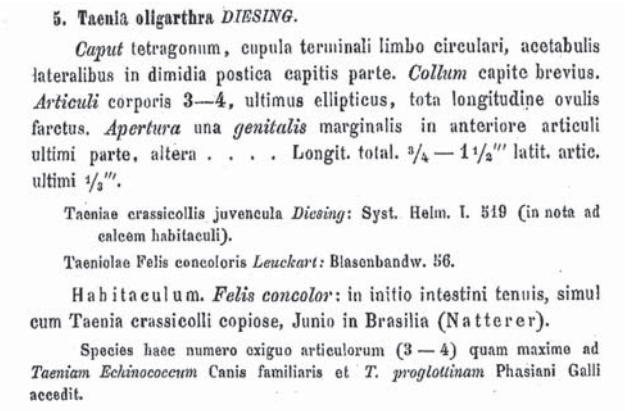
In 1903 and in the years following, Marcelo Viñas in the Buenos Aires province of Argentina described a few cases of what he thought was AE on the American continent. The patients in whom he diagnosed the disease had multilocular cysts with an alveolar aspect, resembling European AE. Notably, the patients came from rural areas and claimed that they had never been out of the country (*4*–*6*). At that time, only *E. granulosus* (described by Batsch in 1786) and *E. multilocularis* were known members of the genus *Echinococcus*. AE had never been detected in South America before and was thought to be restricted to temperate, Holoarctic regions. AE lesions had been recognized as echinococcal 48 years before, in 1855, by Rudolf Virchow (*7*); the causative agent, *E. multilocularis*, had been described by German parasitologist Rudolf Leuckart in 1863 (*8*). The life cycle of the parasite, which involves foxes and rodents, was not elucidated until the 1950s by Robert L. Rausch and Everett L. Schiller (*9*) and Hans Vogel (*10*). Since the patients described by Viñas had never left their home country, he concluded that they must have acquired the disease in Argentina. Would this be the first description of AE in the New World?

Discovery of Adult *Echinococcus oligarthrus*

Many years earlier, on April 9, 1817, the Austrian emperor, Franz I, had sent a group of natural scientists to Brazil to explore the country. On board one of the ships was 36-year-old Johann Natterer (1781–1843), a passionate ornithologist (*11*). In his past search for parasitic worms in birds, Natterer had studied helminthology at the Naturalien-Cabinete of Vienna's Hofmuseum under the supervision of Johann Gottfried Bremser (1767–1827), a physician and helminthologist. Natterer was fascinated by Brazil and stayed abroad for 18 years. He explored the area from Rio de Janeiro to Mato Grosso and British Guyana. Natterer returned to Vienna in 1836 with a Brazilian wife, 3 children, and 37 boxes of collected material (*11*). Among the many specimens he brought home was a helminth he had found in the upper part of the small intestine of a puma, *Felis (Puma) concolor*.

Karl Moritz Diesing (1800–1867), a zoologist and successor to Bremser in Vienna, listed the helminth collected by Natterer in his famous Systema Helminthum of 1850 initially under the juvenile form of *Taenia crassicollis* ("*Taeniolae in fele concolore lectae probabiliter pullae*") found in *F. concolor* (*12*). Rudolf Leuckart (1822–1898) stated in a monograph (*13*) that these helminths may not be seen as juveniles of *T. crassicollis* because they share some characteristics with *T. echinococcus*. Diesing later reclassified Natterer's specimen as *Taenia oligarthra* in his Revision der Cephalocotyleen, which was presented to the scientific academy in Vienna on November 5, 1863 (*14*). In his Latin description, Diesing noted the presence of only 3–4 proglottids (articuli), hence the name "oligarthrus" (Figure 1). Diesing stated that the low number of proglottids is similar to the number of proglottids in *T. echinococcus*. The organism was still not recognized as an echinococcus, however. The presence of hooks typical for echinococci was not mentioned, and the parasite was placed in a subgroup with hookless tapeworms. All of these scientific descriptions of the South American tapeworm were forgotten by 1903, when Viñas described the cases of possible AE in Argentina.

In 1910, Max Lühe (1870–1916), a German physician and zoologist from Königsberg, requested the cestode material from Vienna and extensively characterized the small helminth. Lühe noted that most of the specimens had lost their rostellar hooks but that they were still present in some organisms (Figure 2). He believed that Diesing must have overlooked the few specimens with hooks. Besides the remarkable difference in body length, no discrepancy with *T. echinococcus* was found. Lühe therefore concluded that *T. oligarthra* and *T. echinococcus* were closely related (*15*). Sixteen years later, Thomas Wright Moir Cameron (1894–1980), from the London School of Hygiene and Tropical Medicine, rediscovered the adult tapeworm in a different



5. Taenia oligarthra *DIESING.*

Caput tetragonum, cupula terminali limbo circulari, acetabulis lateralibus in dimidia postica capitis parte. *Collum* capite brevius. *Articuli* corporis 3—4, ultimus ellipticus, tota longitudine ovulis farctus. *Apertura* una *genitalis* marginalis in anteriore articuli ultimi parte, altera . . . . Longit. total. $^3/_4$ — $1^1/_2$‴ latit. artic. ultimi $^1/_3$‴.

Taeniae crassicollis juvencula *Diesing*: Syst. Helm. I. 519 (in nota ad calcem habitaculi).

Taeniolae Felis concoloris *Leuckart:* Blasenbandw. 56.

Habitaculum. *Felis concolor*: in initio intestini tenuis, simul cum Taenia crassicolli copiose, Junio in Brasilia (Natterer).

Species haec numero exiguo articulorum (3 — 4) quam maxime ad *Taeniam Echinococcum* Canis familiaris et *T. proglottinam* Phasiani Galli accedit.

Figure 1. Latin description of adult *Echinococcus oligarthrus* by Karl Moritz Diesing, 1863 (*14,* p. 370). In addition to the morphologic characterization of the helminth, the 2 prior references from Diesing's Systema Helminthum (*12*) and from Leuckart's monography (*13*) are listed. Natterer, who collected the helminth in Brazil, is also mentioned.

South American felid, a jaguarundi (*Felis yaguarondi*), which had died at the London Zoo. Cameron proposed placing *T. oligarthra* in the genus *Echinococcus* (*16*), which had been established by Karl Asmund Rudolphi in 1801. At that time, a cystic larval stage of the parasite had not been found or assigned to a strobilar stage. Whether this parasite could cause human disease was still unknown because no connection to the early Argentinian cases had been established.

Description of the Larval Stage of *E. oligarthrus*

On May 22, 1914, Emile Brumpt (1877–1951) and Charles Joyeux (1881–1966) from the Laboratoire de Parasitologie in Paris autopsied 4 agoutis (*Dasyprocta agouti*, today: *D. leporina*, Figure 3) in the state of São Paulo, Brazil (*17*). In the spleen and liver of one of these South American rodents they found multiple cysts. The liquid of the cysts resembled hydatid sand. The authors stated that the cuticle of the larva was very thin and that this "reminded us that in *Echinococcus granulosus* this cuticle may reach several millimeters." The inner surface of the cysts contained a proliferative membrane with many vesicles and protoscolices, the larval stage of tapeworms. The authors extensively described the protoscolices and the amount and shape of the rostellar hooklets they found. They concluded that the cysts in the agouti resembled the general structure of *E. granulosus* cysts. After comparing the hooks with those from *E. granulosus* and *E. multilocularis*, Brumpt and Joyeux concluded that the larva found in the agouti must have originated from a very small tapeworm. They stated that it was "unfortunately impossible to assign our hydatid to a known adult form." The authors continued to speculate that "due to the origin of the material, it seems

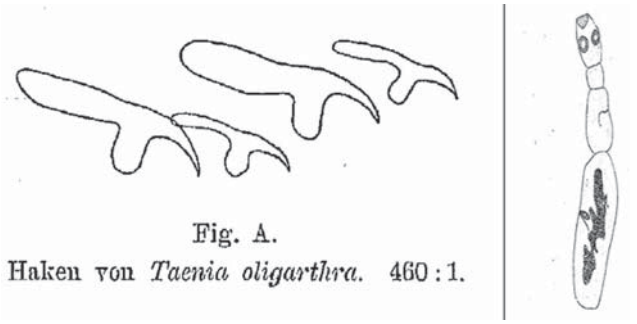


Figure 2. First drawing of the rostellar hooklets (left) and the entire strobilar stage of *Echinococcus oligarthrus* (right). The specimen was listed under no. 396 in the Wiener Hofmuseum. From (*15*).

absolutely indicated to think of *Taenia oligarthra*." However, they concluded that the hooklets previously described by Lühe were different in size and shape and that therefore the cysts in the agouti belonged to a not yet described adult tapeworm, which they tentatively named *Echinococcus cruzi*. Their observations were published 10 years later, in 1924 (*17*).

In 1926, Cameron proposed that *E. cruzi* is the larval stage of *E. oligarthrus*, on the basis of the similar size and shape of the rostellar hooks and their origin in the same geographic region (*16*). Cameron had compared the morphologic features of the helminths' rostellar hooks from the larval stage obtained from the agouti and from the strobilar stage he had rediscovered in the jaguarundi.

Parasite's Life Cycle and Human Infection

Around that time, more cases of the emerging South American PE were recorded by Viñas in Argentina (1932, [*18*]). A single case also occurred in Uruguay and was described by Félix Dévé (1872–1951) and co-workers in 1936 (*19*); a second one was described by G. Dardel in 1955 (*20*). Dévé, a French physician, thought that the new South American echinococcosis was a "forme intermédiaire" between AE and cystic echinococcosis. However, Dévé believed in the unicyst theory of echinococcosis: all types of hydatid disease were caused by a single *Echinococcus* species (*21*,*22*).

In 1966, Vernon E. Thatcher and Octavio E. Sousa from the Gorgas Memorial Laboratory in Panama presented a redescription of adult *E. oligarthrus* on the basis of material from a puma in Panama (*23*). They also implicated humans as possible intermediate hosts, which they deduced from a case report by Sousa and Lombardo Ayala in 1965 (*24*). The latter report described the case of a polycystic, multilocular, hepatic cyst in a native Panamanian; the cyst had characteristics distinct from *E. granulosus* and *E. multilocularis* cysts and was probably caused by a parasite indigenous to the American tropics. The authors concluded that the human hydatid possibly represented *E. oligarthrus*. They further suggested that the polycystic multilocular human hydatidosis of the Panama-Colombia area, studied around that time by Antonio D'Alessandro from the Tulane University International Center for Medical Research in Colombia, might be caused by the same species of parasite.

One year later, adult *E. oligarthrus* was found again by the same authors in the small intestine of another wild felid, the Panamanian jaguar (*Felis* [*Panthera*] *onca*) (*25*). After a reexamination of material previously misconstrued by others, Thatcher and Sousa concluded that a metacestode found in a nutria (*Myocastor coypus*), a South American rodent that had died in a United States zoo, was the larval stage of *E. oligarthrus* (*26*). Until then, various South and Central American felids had been considered to be definitive hosts of *E. oligarthrus,* and the presumed larval stage of the parasite had been discovered in rodents from the same geographic area. Experimental work was needed at that time to elucidate the biologic definition and the life cycle of the parasite. Proof had to be found that the formerly described *E. cruzi* was indeed the presumed metacestode stage of *E. oligarthrus*.

Sousa and Thatcher achieved this aim in 1969 by experimentally inducing hydatidosis in different rodent species. Among others, climbing rats, spiny rats, and agoutis were fed gravid proglottids of *E. oligarthrus* obtained from a naturally infected puma (*27*). In these successfully infected intermediate hosts, mature metacestodes showing similar morphologic features to *E. cruzi* developed in the muscles and inner organs. In a second experiment, the experimentally induced hydatids of the agoutis transformed into adult and mature *E. oligarthrus* in the feline intestine when fed to domestic cats. In return, parasite material obtained from the infected cats produced hydatid cysts in agoutis. In contrast, dogs could not be infected. The house cat was therefore implicated as playing an important role as definitive host and as a potential risk to humans. The life cycle of the parasite, however, was considered to be mainly sylvatic (*27*). After nearly 120 years, the mystery of human PE seemed finally solved. In 1972, however, a second South American species, *E. vogeli*, was discovered.

Discovery of a Second South American Species, *E. vogeli*

In late 1969 or early 1970, Martin Stummer, an animal dealer at Amazon Ltd, a company supplying animals for zoos, captured a bush dog (*Speothos venaticus*) in the province of Esmeraldas in Ecuador. The animal was sent to the Los Angeles Zoo and routinely examined. After a deworming treatment had resulted in the expulsion of numerous cestodes of the genus *Echinococcus*, Calvin Schwabe from the School of Veterinary Medicine in Davis, California, examined the helminths and found unusual morphologic

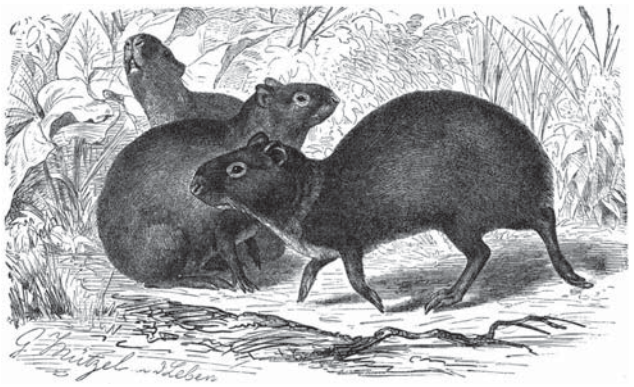


Figure 3. The agouti, *Dasyprocta* sp., one of the natural intermediate hosts for *Echinoccocus oligarthrus*. Drawing by Gustav Mützel (1839–1893).

characteristics. Robert L. Rausch from the Arctic Health Research Center in Fairbanks, Alaska, and J. J. Bernstein from Venice, California, described a small helminth, which differed substantially from all other recognized species of *Echinococcus* (*28*). They named the species *E. vogeli* in recognition of Hans Vogel (1900–1980) from the Bernhard-Nocht-Institute in Hamburg, who contributed to the elucidation of the life cycle of *E. multilocularis*. On the basis of the morphology of the rostellar hooks and other characteristics, Rausch and Bernstein were able to describe this new species in 1972. In the same year, Thatcher concluded that *E. oligarthrus* was likely the cause of all cases of human and animal PE in the neotropics (*29*). However, with the description of a new indigenous species, uncertainties arose about the etiologic role of *E. oligarthrus* in PE (*30*). None of the researchers could know at that time that *E. vogeli* would soon be the most frequently encountered species of the 2 indigenous South American echinococcal tapeworms.

The synonymy of *E. cruzi* with *E. oligarthrus* was then questioned. A reexamination in 1984 of material obtained from Brumpt's and Joyeux' initial case of the agouti demonstrated that the larval stage of *E. oligarthrus* was indeed the causative organism (*31*). In contrast, the metacestode found in the nutria and in the Panamanian patient described in 1965 was shown to be *E. vogeli* (*30*,*32*). The 11 cases described by Viñas in Buenos Aires and those noted by Dévé and Dardel from Uruguay could not be definitively assigned to either *E. oligarthrus* or *E. vogeli*. The presence of protoscolex hooklets, which are used for discrimination, was not described in detail in these reports (*33*). However, the cases are most likely caused by *E. oligarthrus* because the final host of *E. vogeli* is not found in those areas (*33*). By the end of 2007, 3 cases of proven *E. oligarthrus* infection in humans have been reported: 1 cardiac case from Brazil (*34*) and 1 orbital case each from Suriname (*35*) and Venezuela (*36*).

Rausch and Bernstein predicted, on the basis of the known predator-prey relationship of the bush dog, that the larval stage of *E. vogeli* would also occur in rodents, including pacas (*28*). Indeed, parasitic cysts were found in a Colombian paca (*Cuniculus paca*, Figure 4) in 1975. The material was experimentally fed to a dog; in addition, larvae obtained from a Colombian human patient with PE (*37*) were given to a second canid. From both dogs, the strobilar stage of *E. vogeli* was later recovered (*30*). As sufficient material was collected from the field in Colombia and obtained from experimentally infected animals, R.L. Rausch, V.R. Rausch, and A. D'Alessandro were able to morphologically distinguish *E. vogeli* from *E. oligarthrus*. The rostellar hooks of each of the 2 South American species were found to consistently differ in length and form, which permitted discrimination of the tapeworms' larval stages. As a consequence, known human and animal cases of PE were reexamined, and some cases thought to have been caused by *E. oligarthrus* were shown to have been caused by *E. vogeli* instead (*32*). *E. vogeli* typically has a thick laminated outer layer and a thin inner germinal layer, whereas *E. oligarthrus* has the reverse. Calcareous corpuscles are abundant in the germinal layer and in the protoscolices of *E. oligarthrus* but are almost absent in *E. vogeli* (*33*).

In just a few years, a second indigenous South American echinococcal species had been discovered, and the life cycle of the parasite, involving the bush dog and the paca, had been described. In a survey of Colombian mammals, 73 (22.5%) of 325 pacas harbored metacestodes of *E. vogeli*, but only 3 (0.9%) of pacas harbored *E. oligarthrus*. Twenty (6.2%) more pacas were shown to be infected with polycystic larvae, but the species involved could not be determined. In addition to the bush dog, a domestic dog belonging to a hunter was found to be naturally infected with adult *E. vogeli* (*38*). Researchers then assumed that domestic dogs might play a role in the transmission of parasite eggs to humans.

Figure 4. The paca, *Cuniculus paca*, the natural intermediate host for *Echinococcus vogeli* and rarely *E. oligarthrus*. Drawing by Robert Kretschmer (1818–1872).

Current Situation

As of 2007, at least 106 human cases of PE from 12 countries have been documented. The disease occurs exclusively in rural areas of the American tropics and often in regions where *E. granulosus* is not present (*33*). Most cases are reported from Brazil and Colombia (*33*,*39*), but PE is endemic from Nicaragua to Chile (*35*). Its rising frequency (12 cases from 4 countries in 1979, 72 cases by 1997, and 86 cases from 11 countries as of 1998) shows that human PE is an emerging disease and no longer a medical curiosity (*33*). Most cases are caused by *E. vogeli*, but many cases could not be assigned specifically to any of the 2 South American echinococcal species because the presence of hooks was not reported (*33*,*39*). In an advanced laboratory setting, *Echinococcus* species can be distinguished by PCR followed by sequencing or restriction fragment length polymorphism analysis (*40*). Parasite material obtained from those infected, for whom a diagnosis cannot be made by means of classic parasitology, can now be subjected to methods of molecular biology. Why most PE is caused by *E. vogeli* is unclear. Some have speculated that because felids cover their feces, contact with infectious ova of *E. oligarthrus* is less likely than contact with eggs of canid-borne *E. vogeli* (*33*). Accordingly, similar proportions in infection rates of the respective natural intermediate hosts have been found (*38*). Seven species of wild felids that were naturally infected with *E. oligarthrus* have been found. The geographic distribution of wild cats extends from northern North America to southern Argentina. In contrast, the bush dog, the only natural definitive host for *E. vogeli*, is found from Panama to south Brazil. The published number of human cases is probably just the tip of the iceberg (*33*); the true prevalence of human PE is far from being known.

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Atypical Bovine Spongiform Encephalopathies, France, 2001–2007

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In France, through exhaustive active surveillance, ≈17.1 million adult cattle were tested for bovine spongiform encephalopathy from July 2001 through July 2007; ≈3.6 million were >8 years of age. Our retrospective Western blot study of all 645 confirmed cases found that 7 were H-type and 6 were L-type.

Most cases of bovine spongiform encephalopathy (BSE) have shown strikingly uniform features. The origin of epidemics, mainly in the United Kingdom but to a lesser extent in other countries, has been foodborne contamination by a single major strain of the transmissible spongiform encephalopathy (TSE) agent (*1*,*2*). However, in recent years, 2 distinct forms of the disease have been described; these forms deviate phenotypically from the previously identified classic BSE (C-type) (*3*,*4*). Western blot studies of the protease-resistant prion protein (PrPres) showed higher and lower molecular masses of unglycosylated PrPres in these 2 types, subsequently named H-type and L-type compared with C-type BSE (*5*). In L-type BSE, the most discriminant molecular feature was the lower level of diglycosylated PrPres (*4*,*5*). Such cases have now been identified in a number of different countries (*6*–*8*).

The origin of H-type and L-type BSE cases is unknown, but they may represent spontaneous or so-called sporadic forms of TSE, reminiscent of most cases of Creutzfeldt-Jakob disease in humans. Transmission studies in wild-type mice (*9*) or in transgenic mice expressing the bovine PrP gene (*5*,*10*–*12*) have shown that the infectious agents involved in H-type and L-type BSEs differ from the single strain isolated from C-type BSE.

Epidemiologic data are crucial to our understanding of the origin of such cases, but precise prevalence needs to be determined. The prevalence in cattle can now be assessed with accuracy because exhaustive BSE testing of adult cattle was implemented in 2001 in all European member states (January 1 for abattoirs and July 1 for rendering plants). We report the results of a retrospective study to determine the frequency of H-type and L-type BSE identified in France since July 2001.

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The Study

A retrospective Western blot study was performed for all confirmed BSE cases diagnosed in France after July 1, 2001, as previously described (*13*). Briefly, PrPres was extracted using the TeSeE Western blot confirmatory assay (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions. For immunoblotting, antibodies RB1, 6H4 (R-Biopharm, St. Didier au Mont d'Or, France), Sha31 from the TeSeE Western blot (Bio-Rad), and SAF84 (kindly provided by J. Grassi, Commissariat Energie Atomique, Saclay, France) were used; these antibodies recognize the bovine PrP sequences 110–113, 156–164, 156–163, and 175–180, respectively. Two Western blot assays were conducted on the brainstem samples and used either an antibody against the PrP core (RB1, 6H4, or Sha31) or a C-terminal antibody (SAF84) (*13*). The characteristics of H-type and L-type BSE (*6*,*13*) that were sought included 1) a higher or lower molecular mass of unglycosylated PrPres with core antibodies in H-type and L-type BSE compared with C-type BSE, 2) a lower proportion of diglycosylated PrPres in L-type BSE, and 3) presence of an additional band at ≈14 kDa with SAF84 in H-type BSE. All cases with features of H-type or L-type BSE were then subjected to further Western blot analyses for detailed quantitative analysis of PrPres molecular features (apparent molecular masses and glycoforms proportions).

Among the 645 BSE cases confirmed between July 1, 2001, and July 1, 2007, 7 H-type and 6 L-type isolates were identified; these had occurred at a frequency of 0–3 H-type and 1 L-type case per year, compared with 6–219 cases of C-type BSE per year (Table). Molecular typing of 48 of these 645 samples was not possible because low levels of PrPres prevented detailed molecular characterization or because sample amount was insufficient. All 13 atypical cases were detected in cattle >8 years of age, from fallen stock (9 cases) or abattoir (4 cases); not 1 atypical case was found among the 98 BSE cases detected by clinical surveillance during this period. These 13 atypical cases were diagnosed by the different rapid BSE tests routinely used in France: 9 by Prionics-Check Western, LIA, or Priostrip (AES, Combourg, France), 3 by ELISA Bio-Rad, and 1 by IDEXX HerdChek (IDEXX Laboratories, Schiphol-Rijk, the Netherlands). During retrospective interviews, the farmer and veterinarian for 6 of these animals reported clinical signs consistent with TSE in 3 fallen stock. This series of 13 cases identified since the beginning of exhaustive active surveillance should be compared with a total of ≈17.1 million adult cattle tested, of which ≈3.6 million were >8 years of age. In addition to these 13 cases, the first case of atypical (H-type) BSE was identified in 2000, during an exhaustive

Table. Results of retrospective molecular typing studies for types of bovine spongiform encephalopathy (BSE) cases identified, France, 2001–2007*

Year	No. tested	Age >8 y	H-BSE	L-BSE	C-BSE	UC
2001 (Jul–Dec)	1,524,344	334,865	1	0	153	18
2002	3,183,320	648,026	2	1	219	17
2003	3,189,899	649,724	3	1	123	10
2004	2,867,571	614,851	0	1	51	2
2005	2,590,973	565,863	0	1	30	1
2006	2,692,048	555,577	0	1	6	0
2007 (Jan–Jun)	1,070,210	244,286	1	1	2	0
Total	17,118,365	3,613,192	7	6	584	48

*H-type, higher molecular masses of unglycosylated protease-resistant prion protein (PrPres); L-type, lower molecular masses of unglycosylated PrPres; C-type, classic BSE; UC, unclassified.

active surveillance program in rendering plants in a limited region of France.

The distribution of BSE-infected cattle by birth date (Figure 1) shows that 1 or 2 (H- or L-type cases) were eventually found positive by rapid tests of the brainstem in each annual birth cohort from 1986 through 1997, which compares with up to 221 cattle infected with C-type BSE born during 1990–2001 for which BSE was diagnosed during 2001–2007. All H-type and L-type BSE cases showed similar features (Figure 2) regarding the high apparent molecular masses of unglycosylated PrPres (mean difference of ≈1 kDa using 6H4 antibody) for H-type BSE and lower levels of diglycosylated PrPres (mean difference of 35% using 6H4 antibody) for L-type BSE, compared with C-type BSE. For L-type BSE cases, the differences in apparent molecular masses were more obvious for the diglycosylated band (≈1-kDa difference using 6H4, compared with 0.3 kDa for the unglycosylated band), as previously observed (*6*). The ≈14-kDa band characteristic of H-type BSE was similarly detected in the 8 isolates with SAF84 antibody but in none of the other cases classified as L-type or C-type BSE.

Conclusions

This study involved exhaustive molecular typing of BSE cases during a given test period and in a country in which BSE testing has been mandatory for all adult slaughtered or fallen cattle. France tests more animals than any other European country; ≈30% of the animals tested in the European Union are tested in France. The estimated frequency of H-type and L-type BSE was 0.41 and 0.35 per million adult cattle tested, respectively (1.9 and 1.7 in cattle >8 years of age). Given the implementation dates of measures to control BSE and the birth dates of these BSE-infected cattle, foodborne exposure to an infectious agent cannot be fully excluded for any of these cattle. However, the distribution of cattle affected by H- and L-type BSE, by year of birth, differs strikingly from that of cattle affected by C-type BSE and is consistent with the hypothesis that H-type and L-type BSE might represent sporadic prion disorders. In comparison with another sporadic prion disease, the annual frequency of sporadic Creutzfeldt-Jakob disease in humans, which is estimated only by analyses of reported clinically suspect cases, is 1–2 cases per million. Similar studies in other countries, instead of those free of C-type BSE, would be useful. An alternative hypothesis to foodborne contamination, such as contamination by a scrapie agent, cannot be fully excluded, as such contamination has been shown to be a risk factor for scrapie in sheep (*14*).

This study relied on the identification of BSE cases by examination of the brainstem only, as derived from our knowledge of C-type BSE (*15*). We cannot exclude pos-

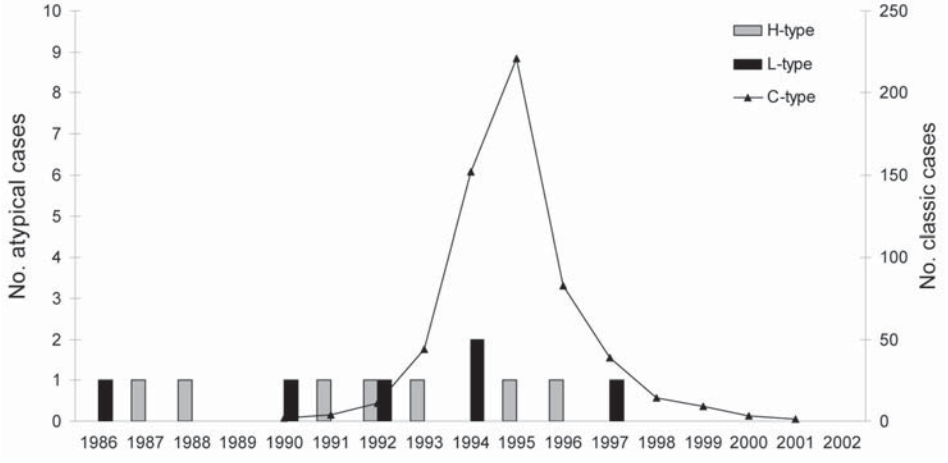

Figure 1. Distribution of bovine spongiform encephalopathy cases identified from July 1, 2001, through July 1, 2007, by year of cattle birth. H-type, higher molecular masses of unglycosylated protease-resistant prion protein (PrPres); L-type, lower molecular masses of unglycosylated PrPres; C-type, classic BSE.

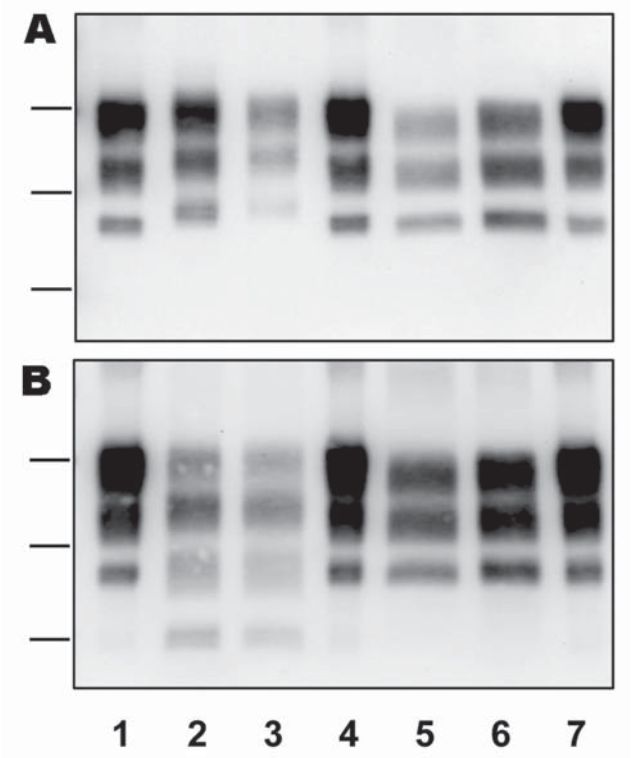

Figure 2. Representative Western blot analyses of protease-resistant prion protein (PrPres) in H-type (lanes 2, 3), L-type (lanes 5, 6), and C-type (lanes 1, 4, 7) cases of bovine spongiform encephalopathy (BSE). Bars to the left of the panels indicate the 29.0-, 20.1-, and 14.3-kDa marker positions. H-type, higher molecular masses of unglycosylated PrPres; L-type, lower molecular masses of unglycosylated PrPres; C-type, classic BSE. Monoclonal antibodies Sha31 and SAF84 were used for PrPres detection in panels A and B, respectively.

sible differences in the pathogenesis of atypical BSEs that might result in underestimation of their frequency, e.g., involvement of the brainstem at a later stage than with C-type BSE. This possibility is at least suggested by data available for L-type BSE, which shows a preferential distribution of abnormal PrP in more rostral brain regions (*4*,*12*). Studies of the pathogenesis of these novel BSE forms are thus important for understanding of prion disorders of domestic ruminant species.

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Burkholderia pseudomallei Antibodies in Children, Cambodia

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Antibodies to *Burkholderia pseudomallei* were detected in 16% of children in Siem Reap, Cambodia. This organism was isolated from 30% of rice paddies in the surrounding vicinity. Despite the lack of reported indigenous cases, melioidosis is likely to occur in Cambodia.

Burkholderia pseudomallei is a soil saprophyte and the cause of melioidosis (*1*). This bacterium can be isolated from soil and water in melioidosis-endemic regions of the tropics, where infection is acquired after bacterial inoculation, inhalation, or ingestion (*1*). Most reported cases occur in Thailand and northern Australia, but this statistic likely represents a fraction of the true extent of disease because microbial culture, the mainstay of diagnostic confirmation, is not available across much of rural Asia. The largest concentration of confirmed melioidosis cases worldwide occurs in northeast Thailand, where the disease accounts for 20% of all community-acquired septicemias (*2*). The death rate for affected adults in this setting is ≈50% (*2*). Northeast Thailand is bordered by Lao People's Democratic Republic (PDR) to the east and southeast and Cambodia to the south. Melioidosis has recently been recognized in Lao PDR after a diagnostic microbiology laboratory was instituted at Mahosot Hospital, Vientiene (*3*), and *B. pseudomallei* has been isolated from the surrounding environment (*4*). By contrast, there are no reports in the literature of indigenous melioidosis or environmental isolation of *B. pseudomallei* in adjacent Cambodia. Two cases of melioidosis have been reported in Cambodian residents in Canada and the United States, respectively; both persons had spent several years in refugee camps in Thailand (*5*,*6*). We propose that melioidosis occurs in Cambodia but is unrecognized because of the lack of diagnostic microbiology facilities. The aims of this study were to conduct a seroprevalence study of children living in Siem Reap, Cambodia, to detect the presence of antibodies resulting from exposure to *B. pseudomallei*, and to determine whether this organism could be isolated from their environment.

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The Study

A prospective, cross-sectional study was conducted at Angkor Hospital for Children, Siem Reap, from December 2005 through April 2006. Unselected consecutive serum samples were collected from children between birth and 16 years of age from the biochemistry and hematology laboratory of Angkor Hospital for Children, Siem Reap. Blood samples were collected from outpatients and inpatients. These blood tests were ordered by the primary physician for other reasons, and the sample used represented surplus material. Samples were centrifuged at 3,000 rpm for 10 min and the serum stored at –30°C. Target sample numbers were 40–60 per year group. An anonymous database was created to record sex, age, and indirect hemagglutination assay (IHA) titer. The presence and titer of antibodies to *B. pseudomallei* were determined by using the IHA, as previously described (*7*), with the exception that the pooled antigens used were from 2 *B. pseudomallei* isolates (strains 001a and 002a) isolated 10 years ago in Phnom Penh, Cambodia. These patients' isolates were sent to us for identification in 1996, although the details of their clinical provenance are unknown. Although data on these isolates are unpublished, their existence suggests the presence of melioidosis in Cambodia. Serum samples were tested in random order. Any detectable IHA titer was interpreted as evidence of exposure to *B. pseudomallei*. Ethical approval for this study was obtained from the Faculty of Tropical Medicine, Mahidol University, Thailand, and the Institutional Review Board Committee of the Angkor Hospital for Children.

Environmental sampling was performed during a 1-day period in January 2006. Four soil samples were collected from each of 10 different paddy fields around Siem Reap. Samples were collected on minor roads leading from route 6 (Thailand to Phnom Penh) up to 80 km southeast and 40 km northwest of Siem Reap. Sampling was performed and *B. pseudomallei* recovered as previously described (*8*). Susceptibility testing was performed by disk diffusion assay for ceftazidime, imipenem, and doxycycline, and by E test for trimethoprim-sulfamethoxazole. Interpretive standards were based on guidelines from the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards).

Serum samples were obtained from 968 children, of whom 528 (54.5%) were male. The number of samples collected per year group is shown in the Figure. Fewer serum samples were collected from children 15 and 16 years of age because only a small number of samples were available from these age groups. A total of 159 children (16.4%) had a detectable IHA titer; values ranged from 10 to 10,240 (median 10, interquartile range [IQR] 20–640).

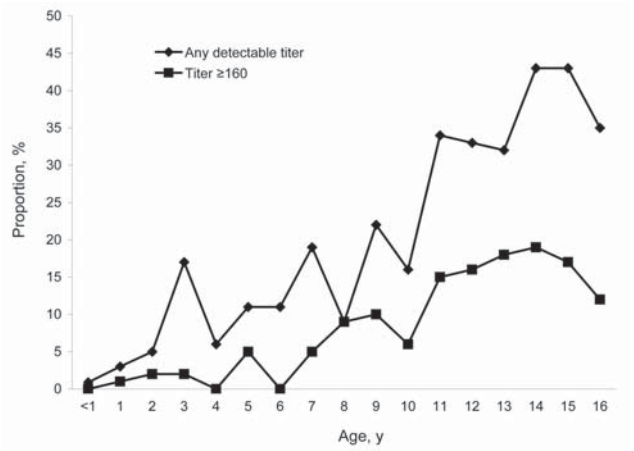

Figure. Indirect hemagglutination assay (IHA) titer for 968 children living in Siem Reap, Cambodia. None of the children was known to have melioidosis. The number of children by age group follows: <1 y, 106 children; 1 y, 98 children; 2 y, 93 children; 3 y, 54 children; 4 y, 50 children; 5 y, 62 children; 6 y, 55 children; 7 y, 57 children; 8 y, 44 children; 9 y, 49 children; 10 y, 50 children; 11 y, 41 children; 12 y, 49 children; 13 y, 66 children; 14 y, 54 children; 15 y, 23 children; 16 y, 17 children.

Females were more likely to have a detectable IHA titer than males (86/440 females [19.5%] vs. 73/528 males [13.8%], p = 0.02), but the distribution of titer values in the population of positive female children was not significantly different from that in the population of positive male children. The proportion of children with any detectable IHA titer rose with age (Figure). An IHA titer ≥160 is used in some centers in Thailand to support a diagnosis of melioidosis in patients with clinical features consistent with this diagnosis. Sixty-three children (6.5%) had an IHA titer ≥160.

B. pseudomallei was isolated from 12 (30%) of 40 soil samples taken in 6 (60%) of 10 rice fields; 1 rice field was positive for all 4 samples, 3 fields were positive for 2 samples, and 2 fields were positive for 1 sample. CFU of *B. pseudomallei* per gram of soil ranged from 1 to 5,000 (median 90 CFU/g, IQR 20–250 CFU/g). *B. thailandensis*, a highly related but usually nonpathogenic organism, was isolated from 1 sample at a concentration of 5,000 CFU/g of soil. All 12 *B. pseudomallei* isolates were susceptible to ceftazidime, imipenem, amoxicillin-clavulanate, chloramphenicol, doxycycline, and co-trimoxazole.

Conclusions

Detection of *B. pseudomallei* antibodies in 16% of Cambodian children is consistent with environmental exposure to this pathogen. The proportion of children with detectable antibodies is lower than that of children in adjacent northeast Thailand (*10*), although the overrepresentation of children in the first 3 years of life in this study could result in a lower comparative figure. The residents of both regions are predominantly agricultural workers and their families, and it seems unlikely that Cambodian children would have a lower level of environmental exposure. The median *B. pseudomallei* colony count of 90 CFU/g soil was lower compared with a reported figure of 230 CFU/g soil in northeast Thailand (*8*), which suggests that the bacterial inoculum present during a given exposure may be lower in Cambodia than Thailand. However, a direct link between environmental bacterial biomass and the rate of seropositivity in the healthy population remains unproven. Seropositivity using IHA is a crude surrogate for good blood culture–supported clinical epidemiology and case detection, although it provides a strong indirect indication of the presence of human melioidosis as a clinically important disease in Cambodia. It also underlines the need for building diagnostic microbiology capacity across rural Southeast Asia.

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Methicillin-Resistant *Staphylococcus aureus*, Geneva, Switzerland, 1993–2005

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Molecular characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) strains different from those of an endemic healthcare-associated clone was conducted over 13 years in Geneva, Switzerland. We demonstrated strain diversity, including clones rarely found in Europe. Local epidemiology of community-associated MRSA is diverse and is evolving by importation and transmission of new strains.

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is responsible for severe infections related to carriage of exotoxins such as the Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin 1 (TSST-1), or exfoliatin A (*1*). Genetic content of CA-MRSA strains depends on the local epidemiology and was recently described as polyclonal (*2*) Another study reported more uniformity in CA-MRSA lineages (*3*). We have recently evaluated prevalence of MRSA at hospital admission and showed a low CA-MRSA prevalence, a reservoir of asymptomatic carriers, and a high degree of CA-MRSA diversity (*2*). However, despite an increasing number of CA-MRSA infections in Europe (*4*), few, if any, studies have assessed long-term epidemiology of CA-MRSA in a geographically confined area. Awareness of secular trends in molecular features of CA-MRSA could affect public health and patient care. Therefore, our aim was to evaluate genetic diversity and spread of non–multidrug-resistant MRSA strains isolated in Geneva over a 13-year period.

The Study

We selected 2 collections of strains with 151 nonduplicated MRSA isolates identified in patients or carriers treated at our institution. The first collection was from a retrospective review of laboratory records and included non–multidrug-resistant (gentamicin- and ciprofloxacin-susceptible) strains collected during 1993–2002 that had a phenotype different from the endemic healthcare-associated MRSA (HA-MRSA) strain in Geneva. The prevalent HA-MRSA clone in Geneva is sequence type (ST) 228-MRSA-I (CC5), which shows resistance to gentamicin, ciprofloxacin, clindamycin, and erythromycin. HA-MRSA strain ST8-MRSA-IV has been sporadically introduced from France. This strain has the same phenotype as ST228-MRSA-I (CC5) except for its susceptibility to gentamicin (*5*). We included all strains resistant to or with intermediate susceptibility to fusidic acid, a characteristic of many CA-MRSA isolates in Europe.

The second collection was isolates selected from patients prospectively identified as colonized or infected with CA-MRSA by the CA-MRSA surveillance program during 2003–2005 (*6*). CA-MRSA was defined as any isolate with an antimicrobial drug resistance profile different from the strain endemic in the Geneva healthcare setting and diagnosed in a patient without a history of hospitalization in the previous 12 months.

MRSA identification was performed by using standard methods (*7*) according to Clinical and Laboratory Standard Institute recommendations (*8*) and confirmed by quantitative PCR (*9*). Genomic DNA isolated from 1 colony was tested by quantitative PCR for staphylococcal cassette chromosome *mec* (SCC*mec*) elements, accessory gene regulator group, and the PVL gene (*10,11*). Presence of type V cassette, TSST-1, and exfoliatin toxins was assessed by using specific oligonucleotides (sequences are available at www.genomic.ch/sup6.php). Multiple-locus variable-number tandem repeat analysis, which consisted of a multiplex PCR with 10 primer pairs, and multilocus sequence typing were performed as reported (*11,12*).

Since late 2002, all patient demographic and epidemiologic data have been reviewed and recorded on a standardized form by a public health nurse (*6*). For this analysis, we included only those patients who were seen at our institution or outpatient clinic.

A total of 92 strains from 51 patients (55% male, mean ± SD age 37 ± 28 years) were obtained from the retrospective specimen collection. Fifty-nine isolates were obtained from clinical specimens and 33 from screening swabs. Among these isolates, 46 were obtained from skin and soft tissue samples and 13 from other body sites. A total of 59 isolates were obtained from the prospective CA-MRSA surveillance system from 59 patients (mean ± SD age 33 ± 21 years, male:female ratio 2.7).

Table 1 shows that most CA-MRSA strains isolated during 2002–2005 were associated with skin and soft tissue infections. Most cases of infection or colonization were associated with migration or travel history. Four healthcare workers acquired CA-MRSA strains epidemiologically unrelated to each other. In 2 instances, family members of these workers were also affected.

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[1]These authors contributed equally to this article.

Table 1. Demographic characteristics, types of infection, and epidemiologic profiles of 61 patients with CA-MRSA colonization or infection, Geneva University Hospitals, 2002–2005*

Characteristic	Value
Demographic data	
Mean ± SD age, y	33 ± 21
Male	35 (57)
Immigrant, foreign origin or residency outside Switzerland	25 (41)
Recent history of travel before CA-MRSA isolation	29 (48)
Institutionalized (prison, nursing home, asylum-seeker camp)	11 (18)
Healthcare worker	4 (7)
Type of infection/colonization	
Primary cutaneous abscess or pyoderma	27 (44)
Wound infection	4 (7)
Impetigo	3 (5)
Other	1 (2)
Colonization	26 (43)
Site of skin infection (n = 27)	
Head and face	6
Upper extremity	6
Trunk and buttock	7
Lower extremity	8
Other clinical features	
Presence of ≥1 other medical condition	16 (26)
Previous exposure (<6 mo) to antimicrobial drug	16 (26)
Case-fatality rate	0

*Values are no. (%) of patients unless otherwise indicated. For 2 patients, no microbiologic specimen was available for molecular characterization. See also supplementary material (available from www.genomic.ch/sup6.php). CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*.

Strains were rarely resistant to clindamycin (5%), gentamicin (8% only in isolates recovered after 2002), or rifampicin (<1%). Susceptibility to cotrimoxazole was 89% during the first period and 100% during the second period. PVL-positive isolates remained multidrug susceptible throughout the study period, and were distinct from our endemic nosocomial strain.

The Figure, panel A, shows the incidence of isolates fulfilling our entry criteria and the proportion of strains producing PVL or harboring SCC*mec* IV or V. An increase in non–multidrug-resistant MRSA was observed during 1994–1997 (incidence 2.3 cases/10,000 admissions in 1997), and a second peak was observed during 2002–2005 (incidence 4.3 cases/10,000 admissions in 2004). Molecular characterization of the 151 strains showed that 124 (82%) harbored either SCC*mec* IV or V. A total of 92 isolates (61%) harbored at least 1 toxin gene, most frequently PVL (n = 60), followed by TSST-1 (n = 22) and exfoliatin A (n = 11). An isolate (ST149-MRSA-IV) from a Libyan patient harbored the PVL and TSST-1 genes (Table 2). A strain with PVL (ST80-MRSA-IV) was isolated in 1994 from a 73-year-old man from Libya. A case of bacteremia with PVL-positive CA-MRSA (ST80-MRSA-IV) was documented in a 28-year-old Tunisian woman who had an abscess of her left forearm in 2000. No case of necrotizing pneumonia was observed.

From 1994 through 1999, we identified 14 PVL-positive MRSA isolates. The Figure, panel B, shows that CA-MRSA identified during 1993–2002 consisted mainly of 3 clonotypes (ST80, ST88, and ST5). After 2002, these strains were less frequent and the proportion of other clonotypes increased.

The online Appendix Figure (available from www.cdc.gov/EID/content/14/2/304-appG.htm) shows that toxin-harboring strains segregated in 18 multiple-locus variable-number tandem repeat analysis profiles and yielded 14 multilocus sequences types. ST80 (n = 39) was the most abundant type (42% of toxin-producing isolates). Other clusters contained well-described ST5 (TSST-1 or PVL

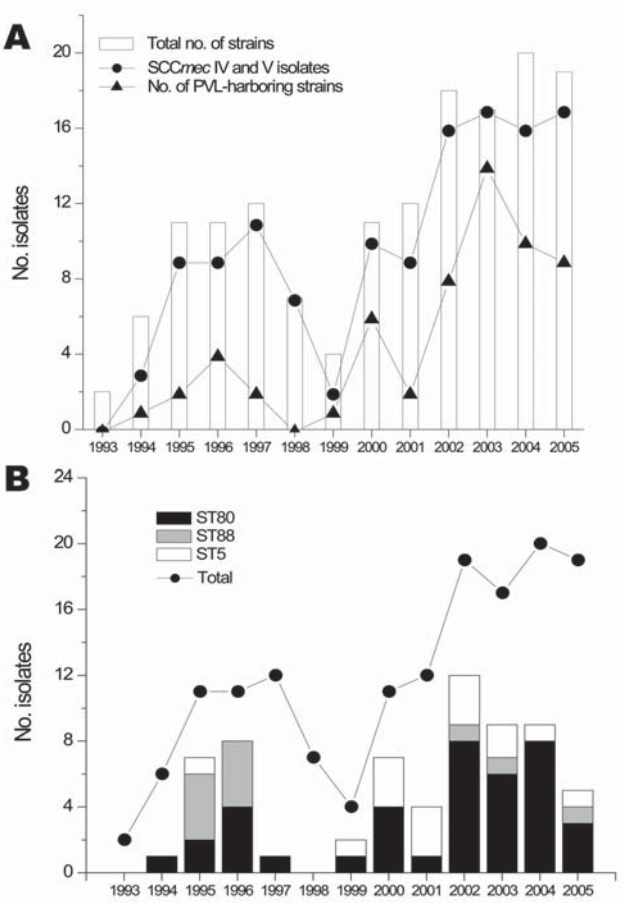

Figure. Incidence of non–multidrug-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) strains, Geneva, Switzerland, 1993–2005. A) Number of strains collected since 1993 showing an atypical multidrug-susceptible phenotype (white bars). Also shown are the number of SCC*mec* IV and V (circles) isolates and number of strains containing Panton-Valentine leukocidin (PVL) (triangles). B) Evolution of the 3 most abundant clonotypes (ST80, ST88, and ST5). Despite a constant number of strains isolated since 2002, the proportion of these clones has decreased, which suggests increasing diversity of clones in our population of community-acquired MRSA.

Table 2. Molecular characteristics of the non–multidrug-resistant methicillin-resistant *Staphylococcus aureus* isolates, Geneva University Hospitals, 2002–2005*

Isolates	No. strains	PVL	Exfoliatin toxin A	TSST-1	*agr* type				Main MLST types
					1	2	3	4	
SCC*mec* I	21	0	0	11	9	11	1	0	5,149
SCC*mec* II	1	0	0	0	0	1	0	0	ND
SCC*mec* III	1	0	0	0	0	0	1	0	ND
SCC*mec* IV†	109	53	11	9	20	24	65	0	1, 5, 8, 22, 30, 72, 80, 85, 88, 149
SCC*mec* V	15	6	0	1	8‡	3	4	0	1, 30, 59, 85, 152
SCC*mec* NT	4	1	0	1	1	1	2	0	ND
Total§	151	60	11	22	38	40	73	0	

*PVL, Panton-Valentine leukocidin; TSST-1, toxic shock syndrome toxin 1; *agr*, accessory gene regulator; MLST, multilocus sequence typing; SCCmec, staphylococcal cassette chromosome mec; ND, not determined; NT, nontypeable.
†Two isolates had recombinases identical to SCC*mec* IVe on the basis of sequence information; 4 isolates were not typeable.
‡One isolate was characterized after PCR amplification and sequencing as previously described (*12*).
§A total of 92 strains had toxins, but the total number of toxin genes detected was 93 because 1 isolate contained PVL and TSST-1.

positive), ST30, and ST8 (USA300) strains harboring the PVL gene.

Several epidemiologically linked cases were identified in the second period (online Appendix Figure): a cluster of 6 family members with recurrent furunculosis over 5 years (ST80-MRSA-IV), 4 smaller family clusters with the same clone, and 3 family clusters with other clonally related strains (ST5-MRSA-IV, ST59-MRSA-V, ST1-MRSA-V). Two inmates incarcerated in the same cell of the Geneva prison had abscesses caused by the ST8-MRSA-IV (USA300) strain. One outbreak involved 5 neonates and 2 mothers colonized or infected with ST5-MRSA-IV harboring the PVL gene (*5*). We also observed a cluster of 5 patients from Kosovo infected or colonized with a PVL-producing strain (ST152-MRSA-V) resistant to gentamicin and amikacin.

Strains lacking toxins (online Appendix Figure, panel B) showed a wide diversity of patterns. Most isolates (n = 33) were obtained from the first stain collection and showed many different genetic backgrounds (n = 21).

Conclusions

We studied 2 collections of non-multiresistant MRSA strains identified over a 13-year period at our institution. Our analysis showed that sporadic PVL-positive CA-MRSA has been isolated in Geneva since 1994; the largest cluster corresponded to ST 80 (SCC*mec* IV, PVL positive); the PVL gene is disseminated in many genetic backgrounds; strains showed diversity of genomic content; several epidemiologic clusters were identified; and many cases were linked to migration and travel.

Our data showed that resistance to fusidic acid or susceptibility to gentamicin should not be used as phenotypic criteria for CA-MRSA in Europe. For example, gentamicin-resistant ST152-MRSA-V found in 5 patients from Kosovo is common.

The number of genetic profiles identified appears particularly high, which is similar to profiles described in European countries, northern Africa, Oceania, and the Americas. In contrast to CA-MRSA epidemiology described as homogeneous in Australia (*13*), some parts of the United States (*14*), or Sweden (*15*), our local epidemiology appears more heterogeneous.

Frequently observed importation of CA-MRSA may enhance genetic exchange between strains and supports the need for active surveillance. Most CA-MRSA remained susceptible to many antimicrobial drugs, but genetic exchange between strains resulting in acquisition of resistance determinants in CA-MRSA or transfer of virulence markers into HA-MRSA are important concerns.

Our study has limitations. First, we used 2 strain collections. The collection obtained before 2003 may have omitted gentamicin-resistant CA-MRSA strains (e.g., ST152). Second, retrospective case ascertainment does not distinguish invasive from colonizing strains in all patients. Finally, we cannot exclude detection bias caused by our active MRSA screening policy (*6*,*7*).

In summary, increasing incidence of PVL-producing type IV CA-MRSA isolates is worrisome and indicates emergence of new MRSA lineages with a particular fitness for community transmission. Further epidemiologic and molecular typing studies are needed to document CA-MRSA carriage and infection rates and implement adequate infection control guidelines.

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Experimental Infection and Natural Contact Exposure of Dogs with Avian Influenza Virus (H5N1)

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Experiments that exposed influenza virus (H5N1)–infected cats to susceptible dogs did not result in intraspecies or interspecies transmission. Infected dogs showed increased body temperatures, viral RNA in pharyngeal swabs, and seroconversion but not fatal disease.

Highly pathogenic avian influenza (HPAI) virus (H5N1) has spread across Asia, Europe, and Africa. Transmission of the virus to felids has been repeatedly reported (*1*–*4*). Investigations also indicate virus transmission to dogs. A fatal infection was documented in Thailand (*5*,*6*). In central Thailand, seroprevalence of ≈25% among 629 village dogs was reported (*7*). The virus was also detected in 2 dogs on Bali (*8*). The often close contact between dogs and humans raises questions about the zoonotic potential and the role of dogs in transmission and adaptation of influenza virus (H5N1) to mammals.

The Study

Experiments were performed on 2 groups of animals housed in different rooms in the high-containment animal facility (Biosafety Level 3+) at the Friedrich-Loeffler-Institut using the highly pathogenic influenza virus (H5N1) strain A/cat/Germany/R606/2006 (*2*,*9*). All experiments were approved by the ethics committee. The first group comprised 5 dogs and 3 cats; 3 cats and 3 dogs were in the second group. The dogs (beagles, 10–12 weeks of age) were obtained from Harlan Laboratories (Borchen, Germany). The cats (8–10 months of age) were obtained from Charles River Laboratories (Dublin, Ireland). All animals were seronegative to influenza by ELISA (Pourquier Blocking AI type A (Rhone Mtrieux, France) and negative to influenza H5 antigen in hemagglutination-inhibition assays (HI).

In the first group of animals, 4 dogs were inoculated oculo-nasopharyngeally with 10^6 50% egg infectious dose (EID_{50}). From day 1 postinfection (p.i.) onwards, 1 uninfected dog and 3 uninfected cats were housed in the same containment room. The cats had the possibility of withdrawing and hiding, but they could also have direct contact with the dogs through 1 part of the cage fence. During the study the cats frequently had direct nose-to-nose contact with the dogs. For a realistic contact exposure setting, the cats were fed by using the dogs' food and water bowls without prior cleaning. In the second group, 3 cats were inoculated oculo-nasopharyngeally with 10^6 EID_{50}. Three uninfected dogs were housed in the same containment room. Direct contact between the animals was similarly enabled as for group 1, and contact again occurred frequently. Two cats and 2 dogs housed in a separate room served as negative controls. Animals were monitored by physical examination and for viral excretion for 21 days by using pharyngeal and rectal swabs.

Conjunctivitis and elevated body temperatures (39.2°C to 39.7°C) developed within 2 days p.i. in all inoculated dogs (group 1). On day 4 p.i., the conjunctivitis had resolved and only 2 dogs had body temperatures >39°C. By day 6 p.i., body temperatures of all animals had declined to <39°C. No additional clinical signs were observed.

Viral RNA was detected in pharyngeal and rectal swabs by real-time reverse transcriptase–PCR (RT-PCR), according to the method of Spackman et al. (*10*). Infectious virus was detected by titration of swab fluid in MDCK cells. Among the pharyngeal and rectal swabs taken at days 0, 2, 4, 6, and 18 p.i., only the pharyngeal swabs taken on day 2 p.i. from 3 of the inoculated dogs were positive by RT-PCR. No infectious virus was isolated. Plasma and peripheral blood mononuclear cell (PBMC) samples taken on day 4 p.i. were negative for viral RNA.

One negative control dog and 2 inoculated dogs were euthanized on day 10 p.i. Sera derived from these animals were negative in the ELISA and HI tests. The serum of 1 dog euthanized on day 21 p.i., however, was positive in ELISA and in HI testing with a titer of 16. Pharyngeal swabs from this dog were also positive by RT-PCR (Table). At necropsy, no gross lesions were present that could be attributed to the influenza infection. Histopathologic examination of the liver showed a scant lymphocytic periportal infiltration in dog no. 3. Influenza virus nucleoprotein could not be detected by immunohistochemical tests in trachea, lungs, liver, kidney, adrenals, thyroid, spleen, lymph nodes, or thymus. Antibodies did not develop in the second dog, euthanized on day 21 p.i. This animal also never tested positive for viral RNA in swab samples by RT-PCR.

Sera of the inoculated dogs were investigated biochemically for all enzymes that could be analyzed with a FUJI DRI-CHEM 3500 i (Sysmex, Leipzig, Germany). Elevated liver enzymes have been reported in cats that were naturally infected with influenza virus (H5N1) (*2*). Two dogs showed

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Table. Course of experimental influenza virus (H5N1) infection of dogs and in-contact animals*

Dog no.	EID_{50}	Body temperature (dpi)				Pharyngeal swab (dpi)				Euthanized (dpi)	Antibodies	
		0	2	4	6	0	2	4	6		HI	ELISA
1	10^6	38.5	39.2	38.8	38.8	–	+	–	–	10	–	(+)†
2	10^6	38.4	39.7	39.1	38.8	–	+	–	–	10	–	(+)‡
3	10^6	38.5	39.3	38.6	38.4	–	+	–	–	21	16	+
4	10^6	38.7	39.3	39.1	38.6	–	–	–	–	21	–	–
5 (dog contact)	0	38.3	38.4	38.0	38.7	–	–	–	–	21	–	–
6 (cat contact)	0	38.4	38.2	38.4	38.5	–	–	–	–	21	–	–
7 (cat contact)	0	38.3	38.5	38.6	38.8	–	–	–	–	21	–	–
8 (cat contact)	0	38.4	38.5	38.2	38.3	–	–	–	–	21	–	–
9 (neg. control)	0	38.4	38.2	38.7	37.8	–	–	–	–	21	–	–
10 (neg. control)	0	38.3	38.6	38.8	38.0	–	–	–	–	21	–	–

*Elevated body temperatures (above 39°C) are underlined. Real time reverse transcription–PCR results are given as – (cycle threshold [ct] values ≥38) and + (ct values 31–38). Animal serum samples were examined by hemagglutination-inhibition (HI) using highly pathogenic influenza virus (H5N1) strain A/cat/Germany/R606/2006 (*9*) as antigen. Results are given as reciprocal titers. Competitive nucleoprotein ELISA results are given as – (inhibition <65%) and + (inhibition ≥65); EID_{50}, 50% egg infectious dose; dpi, days postinfection.
†Serum from dog no. 1 on dpi 10 showed 39.4% inhibition.
‡Serum from dog no. 2 showed 21.6% inhibition.

increased aspartate aminotransferase (AST) values up to 110 U/L (reference values 33–52 U/L) on day 4 p.i. These dogs also showed elevated body temperature on days 2 and 4 p.i., and viral RNA was detected in pharyngeal swabs on day 2 p.i. Dog no. 2 (Table) also showed elevated creatine phosphokinase (CPK) values up to 441 U/L (reference values 54–361 U/L) on day 4 p.i. The cause of the elevated AST and CPK levels noted on day 4 p.i. could have been nonspecific muscle injury. This dog was euthanized on day 10; no muscle injury was observed at necropsy.

The uninoculated dog housed with the infected dogs, as well as the 3 uninfected contact cats in group 1, never showed clinical symptoms. None of the pharyngeal and rectal swab samples, PBMC, or sera derived from these contact animals was positive by RT-PCR. No specific antibodies were detected. Clearly, the virus was not transmitted to the contact dog and cats.

In the second group of animals, severe symptoms—including high body temperatures (≥40°C), decreased activity, conjunctivitis, and labored breathing within 2 days p.i. similar to recent reports about influenza virus (H5N1) infections of cats (*11,12*)—developed in all infected cats. The cats excreted virus through the respiratory and digestive tract. Viral titers quantified in the pharyngeal swabs reached 50% tissue culture infectious dose 10^5 between days 2 and 4 p.i. Two animals were euthanized because of their symptoms within 5 days p.i. The third infected cat recovered and had HI titers of 64 within 2 weeks p.i. No clinical symptoms developed in the 3 contact dogs, and pharyngeal swabs, PBMC, and sera were negative by RT-PCR. Specific antibodies did not develop in the dogs (Table).

Conclusions

Dogs are susceptible to HPAI virus (H5N1) infection. In our study, they reacted with a transient rise in body temperature and in some instances with specific antibodies. Viral RNA was detected in pharyngeal swabs. Infectious virus could not be reisolated, and transmission of virus to a contact dog and cats did not occur. Contact exposure experiments of influenza virus (H5N1)–infected cats with uninfected dogs did not result in interspecies transmission. The different outcome of infection with the same dose of influenza virus (H5N1) suggests that cats are more susceptible than dogs to disease. However, the experiments were performed with healthy animals; concurrent infections, impaired immune functions, and changing viral characteristics might lead to aggravated infections. Also, since some dog breeds are genetically predisposed for certain viral and bacterial diseases, other breeds might be more susceptible to influenza virus (H5N1) infection (e.g., equine influenza virus [H3N8] caused disease predominantly in small groups of dogs of particular breeds, including greyhounds [*13*]). Therefore, dogs may have a role in adaptation of HPAI virus (H5N1) to mammals and its subsequent transmission to humans.

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Timeliness of Enteric Disease Surveillance in 6 US States

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We reviewed timeline information for a sample of *Salmonella* spp.*, Shigella* spp.*, Campylobacter* spp., and *Escherichia coli* O157:H7 cases and all confirmed foodborne outbreaks reported in 6 states during 2002. Increasing the timeliness of case follow-up, molecular subtyping, and linkage of results are critical to reducing delays in the investigation of foodborne outbreaks.

Timely reporting of foodborne diseases is necessary to identify persons at risk for exposure and to prevent additional cases in outbreak settings (*1*). The present study assesses time intervals for surveillance of foodborne diseases and investigation of outbreaks. Results establish baseline measures to evaluate foodborne disease surveillance systems and identify strategies for improvement (*2–4*).

The Study

Data on case investigation timelines in 2002 were collected from records at state and local health departments and public health laboratories in each of 6 states for ≤100 *Salmonella* spp. isolates, ≤50 *Shigella* spp., *Escherichia coli* O157:H7, and *Campylobacter* spp. isolates, and for all foodborne outbreaks. Participating states included 1 with a large population (>6 million), 3 with a medium-sized population, and 2 with a small (<2 million) population from 5 different geographic regions. Two states received supplemental funding through FoodNet. Rules mandated reporting of diagnosed cases from physicians or clinical laboratories to local health departments (2 states), to the state health department (2 states), or to both (2 states). Cases were selected by systematically choosing every *n*th record on the basis of the number of cases reported and the number sampled.

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For 1,319 cases, dates were collected for the following: onset of symptoms (873 [66%]), stool specimen collection (1,088 [82%]), culture result (633 [50%]), report to state or local health department (553 [42%]), submission of isolate to public health laboratory (882 [98%] of 899 isolates that were submitted), case interview (648 [49%]), and molecular subtyping by pulsed-field gel electrophoresis (PFGE) (634 of 635 isolates that were subtyped). Although stool culture result dates were recorded for 633 cases, most were for final culture results based on confirmation by the public health laboratory. Thus, initial culture result dates were available for 147 (11%) cases. For each case, intervals between milestones were calculated from the dates available.

For 112 outbreaks of foodborne disease, dates were collected for the following: implicated meal or event (100 [89%]), onset of symptoms of index case-patients (112 [100%]), first stool collection (65 [79%] of 82 outbreaks for which stool samples were collected), foodborne illness complaint or report of outbreak-related case to health department (99 [88%]), initiation of outbreak investigation activities (90 [80%]). For each outbreak, intervals were calculated from the dates available.

The median intervals from onset of symptoms to surveillance milestone events for individual cases were as follows (Table 1): collection of stool samples, 2–4 days; initial stool culture results, 5–8 days; case report to health department, 7–9 days; isolate submission to public health laboratory, 8–10 days. For case-patients who were interviewed, the median interval from onset of symptoms to interview was 12 days for *E. coli* O157:H7 cases, 14 days for *Salmonella* spp. and *Shigella* spp. cases, and 18 days for *Campylobacter* spp. cases. For isolates that were subtyped by PFGE, the median intervals from onset of symptoms to subtyping were 15 days for *E. coli* O157:H7, 18 days for *Salmonella* spp., and 21 days for *Shigella* spp.

A higher percentage of isolates were submitted to the public health laboratory in states where submission was required (98% for *Salmonella* spp. isolates, 100% for *E. coli* O157:H7) compared to states where submission was not required (75% for *Salmonella* spp. isolates, 80%

[1]The Enteric Disease Investigation Timeline Study Work Group consisted of Patrick McConnon, Knachelle Hodge, Jennifer Lemmings, Jesse Greenblatt, Bela Matyas (Council of State and Territorial Epidemiologists); Richard Skibicki, Donald Sharp, Arthur Liang, Stuart Capper, Stephen Monroe, Kelley Hise, Cindi Snider, Vince Radke, Carol Selman (Centers for Disease Control and Prevention); Brian Collins, Tom Dickey (National Environmental Health Association); John Besser, Veronica Malmberg (Association of Public Health Laboratories); Rob Blake, Rebecca Shapack (National Association of County and City Health Officials); John Lattimore, Sarah Pichette (US Food and Drug Administration); Fred Ramsey (US Department of Agriculture).

Table 1. Median number of days from onset of symptoms to specified timeline event for reported *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *Escherichia coli* O157:H7 infections, 6 US states, 2002

Timeline event	Median no. days after symptom onset			
	Salmonella spp.	*Shigella* spp.	*Campylobacter* spp.	*E. coli* O157
Collection of stool sample	4	2	3	3
Stool culture result	7	6	8	5
Case report from clinician to health department	9	8	9	7
Submission of isolate to public health laboratory	10	8	10	8
Case interview	14	14	18	12
PFGE* subtyping	18	21	Not routinely performed	15

*PFGE, pulsed-field gel electrophoresis.

for *E. coli* O157:H7). However, no difference was found between these states in length of time for isolates to be submitted.

Of 112 confirmed foodborne disease outbreaks, 83 (74%) had an etiologic agent confirmed by laboratory testing (Table 2) (5). Of 29 outbreaks that were not confirmed, norovirus was the suspected cause in 17 (59%) outbreaks, and toxigenic bacteria were suspected in 7 (24%) outbreaks. Median intervals from onset of symptoms to outbreak complaint or recognition were 1 day for bacterial toxins, 3 days for norovirus, 8 days for *E. coli* O157:H7 and *Campylobacter* spp., and 16 days for *Salmonella* spp. (Table 2). Overall, 83 (74%) outbreaks were detected by a consumer complaint, 12 (11%) were detected by a healthcare provider, 11 (10%) were detected by PFGE cluster evaluation, and 6 (5%) were identified through an interview with an individual case-patient. Intervals from onset of symptoms to consumer complaint (median 3 days, range 0–21 days) or to report by healthcare provider (median 3 days, range 0–11 days) were similar. Outbreaks identified by case interview (median 11 days, range 6–16 days) or PFGE cluster evaluation (median 23 days, range 7–83 days) followed case surveillance timelines described above. The median interval from detection of the outbreak to the initiation of the first outbreak investigation step was 0 days (range 0–41 days) for all outbreaks.

The median duration of exposure for all outbreaks with a confirmed etiologic agent was 1 day (range 1–21 days). However, 12 (29%) of 41 norovirus, 2 (67%) of 3 *E. coli* O157:H7, and 9 (75%) of 12 *Salmonella* spp. outbreaks occurred over multiple days. The median duration of multiday outbreaks was 4 days for norovirus (range 2–13 days), 5 days for *E. coli* O157/H7 outbreaks (range 5–6 days), and 10 days for *Salmonella* spp. outbreaks (range 3–21 days).

Conclusions

The multiple steps between onset of a foodborne illness and its investigation by a public health agency result in delayed recognition of outbreaks caused by reportable enteric diseases. One important way to speed the detection of outbreaks is to encourage clinicians to immediately notify health departments when they suspect a patient is part of an outbreak. Since many outbreaks caused by *E. coli* O157:H7 and *Salmonella* spp. last multiple days, physician reporting concurrent with stool collection may provide opportunities for a public health intervention that could prevent outbreak-associated cases.

The speed with which clinical laboratories receive, process specimens, and report results varies by setting, agent, and location. The lack of detail available about these steps is an important limitation of this study. However, health departments generally receive reports from clinicians a median of 2 days after the culture result, and isolates are submitted to public health laboratories within 2–3 days of the initial culture result. These data suggest that improving physician and laboratory reporting practices and logistics could shorten the reporting timeline by 1 or 2 days for most cases.

Timeline elements directly under control of public health agencies include the interval from case report to interview and from submission of the isolate to subtyping by PFGE. Our results demonstrate more variability for these intervals than for earlier steps in enteric disease surveillance. In particular, *E. coli* O157:H7 infections appear to

Table 2. Median number of days from onset of symptoms to outbreak detection for outbreaks with confirmed etiology, 6 US states, 2002

Confirmed etiologic agent	No. (%) outbreaks with confirmed etiologic agent	Median no. days from onset of symptoms to outbreak detection (range)
Salmonella spp.	20 (24)	16 (2–83)
Campylobacter spp.	3 (4)	8 (7–9)
Escherichia coli O157:H7	4 (5)	8 (7–18)
Norovirus	44 (53)	3 (0–11)
Bacillus cereus, Staphylococcus aureus, and *Clostridium perfringens*	10 (12)	1 (0–3)

receive a higher priority than *Salmonella* spp.*, Shigella* spp.*, or Campylobacter* spp. infections. Half of *E. coli* O157:H7 cases but less than one fourth of *Salmonella* spp. cases were contacted by a local health department on the same day the report was received. In addition, outbreaks caused by *E. coli* O157:H7 were detected a median of 8 days sooner than outbreaks caused by *Salmonella* spp. Given the risk for hemolytic uremic syndrome after *E. coli* O157:H7 infections and the potential for person-to person transmission, such attention is warranted. Even so, the intervals from onset of symptoms to PFGE subtyping documented in the nationwide outbreak of *E. coli* O157:H7 infections associated with spinach demonstrated that little has changed across the public health system from 2002 to 2006 (*6*). This and other widespread outbreaks of *Salmonella* spp. infection reinforce the need to increase the timeliness of case follow-up, molecular subtyping, and the linkage of results between them that can reduce delays in the investigation of foodborne outbreaks (*7*).

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Dengue Virus 3 Genotype 1 Associated with Dengue Fever and Dengue Hemorrhagic Fever, Brazil

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Dengue serotype 3 viruses were isolated from patients in Brazil from 2002 through 2004. On the basis of phylogenetic analyses, these isolates were assigned genotype 1. This genotype had never been reported in South America before. Its appearance indicates a major risk factor for dengue epidemics and severe disease.

Currently, dengue is the most significant mosquito-borne viral disease that affects humans. *Dengue virus* (DENV) is transmitted to humans by *Aedes aegypti* mosquitoes; for most persons, this infection is either asymptomatic or dengue fever (DF) develops. In a few cases, DF can progress to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Epidemiologic and phylogenetic studies indicate that particular DENV strains are more virulent than others (*1*,*2*). DENV comprises 4 serotypes. Phylogenetic and molecular analyses showed extensive variability among the DENV serotypes, which led to the recognition of different genotypes within each serotype. For DENV-1 and DENV-2, five genotypes have been described (*2*); DENV-3 and DENV-4 have been subdivided into 4 and 2 genotypes, respectively (*3*,*4*). Regarding DENV-3, genotype 1 includes isolates from Southeast Asia and the South Pacific islands; genotype 2, Thailand; genotype 3, the Indian subcontinent, East Africa, and a single isolate from Samoa; and genotype 4, Puerto Rico and Tahiti (*3*).

Since 2000, DENV-1, DENV-2, and DENV-3 have been found cocirculating in 22 of the 27 states in Brazil (*5*,*6*). DENV-3 in Brazil belongs to genotype 3 and includes strains from Sri Lanka, India, and Africa (*6*–*8*). Since 1996, successive epidemics have been occurring in the city of Belo Horizonte (estimated population 2,214,000), which is located in the south-central region of Minas Gerais State, Brazil. Of the 700,000 dengue cases reported in Latin America in 1998, 12.4% were from Belo Horizonte, and 58.8% were from Minas Gerais State, respectively (*9*). Besides DENV-1 and DENV-2, the DENV-3 serotype was also detected in Minas Gerais State, but only a few DENV-3 isolates have been analyzed with respect to their genetic variability.

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The Study

We analyzed the C-prM and the envelope genes of DENV-3 isolates related to different clinical manifestations of dengue disease from Minas Gerais State, Brazil, from 2002 through 2004. Nine acute-phase serum samples from patients with DF or DHF (previously identified by PCR as DENV-3) were selected for this study (Table). All serum samples were from patients living in the city of Belo Horizonte or neighboring cities (Figure 1). Only 1 case-patient (patient MG-20) died.

For viral isolation, 50 μL of each serum sample was incubated with C6/36 cells, and at least 3 successive passages were conducted for each sample. Microscopic examination of cells inoculated with serum from the patients showed a clearly visible cytopathic effect with changes in the monolayer such as syncytial cell formation and cytoplasmic vacuoles after the third passage (data not shown).

Supernatants of infected C6/36 cells showing typical cytopathic effect were used for viral RNA extraction (QIAamp Viral RNA Kit, QIAGEN, Inc., Valencia, CA, USA). RNA was used as template in reverse transcription–PCR (RT-PCR), as described (*10*).

For determination of nucleotide sequences in the C-prM region from 9 virus samples, the amplicons were cloned into a pGEM-T vector (Promega Corp., Madison, WI, USA), and 3 clones for each isolate were used in sequencing reactions. To determine the nucleotide sequence of the envelope gene from 4 isolates, we purified PCR amplicons (QIAquick Gel Extraction Kit, QIAGEN) and directly used in sequencing reactions. Each DNA sample was sequenced at least 3 times in both orientations (MegaBACE sequencer, GE Healthcare, Buckinghamshire, UK). Nucleotide sequences were aligned with other DENV-3 sequences. The midpoint rooted phylogenetic trees were constructed by the neighbor-joining method with 1,000 bootstrap replicates using the Tamura Nei model implemented by the software MEGA 3.1 (Arizona State University, Phoenix, AZ, USA).

Sequence comparisons showed high degrees of identity among our isolates, and the paired identity at the nucleotide level ranged from 99.2% to 100% and from 99.7% to 99.9%

Table. Description of the dengue virus serotype 3 isolates*

Identification	Disease	Patient sex	Patient age, y	City	Year	Passage history†	GenBank accession nos.	
							E sequence	C-prM sequence
BH–16	DF(HM)	M	42	Belo Horizonte	2003	5	EF625832	EF428575
BH–17	DF(HM)	M	6	Belo Horizonte	2003	4		EF428567
BH–19	DF	M	29	Belo Horizonte	2003	5	EF625833	EF428574
MG–20‡	DHF	F	18	Contagem	2004	4	EF625835	EF428572
MG–21	DF	F	37	Sabará	2003	5		EF428568
BH–22	DHF	F	11	Belo Horizonte	2004	5		EF428571
BH–24	DF	F	37	Belo Horizonte	2003	6	EF625834	EF428570
BH–25	DF	M	25	Belo Horizonte	2003	3		EF428569
MG–27	DF	–	–	Caetanopólis	2002	6		EF428573

*DF, dengue fever; DF(HM), dengue fever with hemorrhagic manifestations; DHF, dengue hemorrhagic fever, according to World Health Organization definition; –, data not available.
†Number of passages in cell culture (C6/36 cell line) is shown.
‡Fatal case.

regarding the C-prM region and the envelope gene, respectively. When sequences were compared with genotype 3 isolates, including isolates from Latin America, the Indian subcontinent, and East Africa, the identity values ranged from 95.4% to 96.2% and from 94.0 to 95.6% in relation to the C-prM and envelope genes, respectively. When those were compared to genotype 1 sequences from the Philippines and China, the nucleotide identities of C-prM region ranged from 98.4% to 99.4%; when envelope sequences were analyzed, values from 99.1% to 99.5% nucleotide identity were observed. According to phylogenetic clustering with other DENV strains (Figure 2), viruses were classified into a specific serotype and genotype. Both phylogenetic trees showed isolates from Brazil grouped together in a well-supported distinct cluster of genotype 1 isolates.

Conclusions

Various genomic regions of DENV have been used for molecular phylogenetic analyses. As described (*10,11*), the C-prM junction and envelope genes have been used as the most sensitive method for virus detection because they harbor epidemiologically relevant sequence information.

Although consensus nucleotide sequences of DENV isolated from different localities have provided some measure of genetic diversity, only a small number of studies use viruses isolated from the same location. Moreover, phylogenetic studies indicated an association between specific genotype and the severity of the disease (*8,12*).

By analyzing a conserved region of the DENV genome (504 nt of C-prM gene) and a more variable region (1,023 nt of the envelope gene), we verified that the DENV-3 isolates belong to genotype 1 (Figure 2). Other DENV-3 isolates sampled from Rio de Janeiro, Brazil, from 2001 to 2002 during an outbreak and also in Latin America were assigned to genotype 3, which has been associated with DHF outbreaks (*6–8,13*).

Phylogenetic studies have shown that DENV can move long distances between continents as well as short distances between neighboring countries (*8,14*). In this study, all DENV-3 isolates, which were associated with DF, DHF, and a fatal case, belonged to genotype 1. However, no consistent differences among the isolates in relation to C-prM or envelope sequences were found to be associated with distinct clinical outcomes nor did they form phylogenetically distinct groups. If the disease severity in DENV-3–infected patients does have a genetic basis, it cannot be attributed to the C-prM or envelope gene.

DENV-3 genotype 1 in Minas Gerais State is more likely to be imported from Asia because these isolates were closely related in the phylogenetic tree to the reference strain from the Philippines and China (*15*). It is difficult to determine the precise route of importation of DENV into Minas Gerais because the available data are limited. But one could speculate that the introduction of genotype 1 into Minas Gerais occurred in 2002, because this genotype had not been detected in that area before 2002. This study shows the emergence of a new DENV-3 genotype not only in Minas Gerais, but in the entire subcontinent, which is a matter for prospective public health studies.

Figure 1. Location of the city of Belo Horizonte and its boroughs Pampulha, Nordeste, and Noroeste, Minas Gerais State, where samples of dengue virus serotype 3 viruses were collected during 2002–2004.

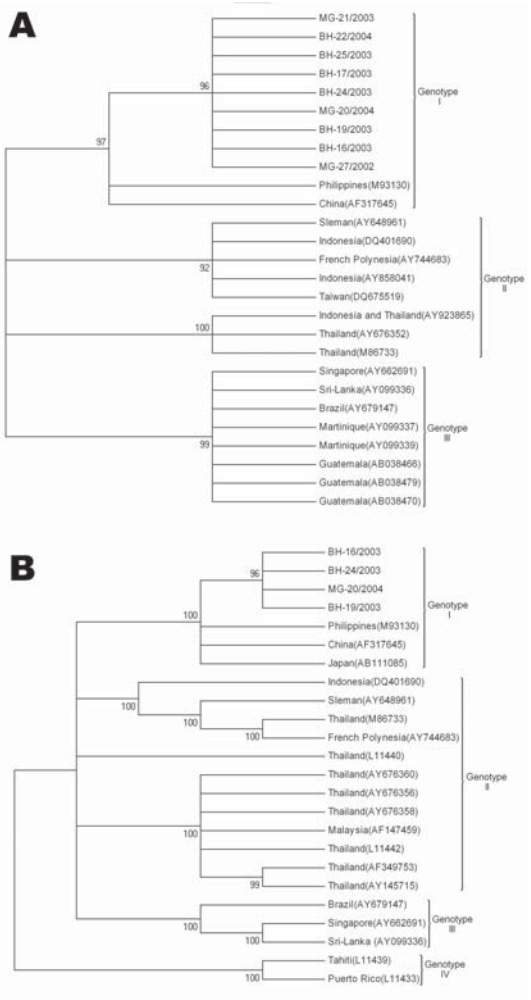

Figure 2. Phylogenetic trees of established dengue virus serotype 3 (DENV-3) and new sequences from Minas Gerais State, Brazil, identified in this study. A) The tree is based on a 504-nt sequence alignment comprising the C-prM gene (nucleotides 137–638). B) The tree is based on a 1,023-nt partial E nucleotide sequences (nucleotides 1022–2008). This tree was generated by neighbor-joining using the Tamura Nei model implemented by using MEGA3 software (www.megasoftware.net). Numbers to the left of nodes represent bootstrap values (1,000 replicates) in support of grouping to the right. Numbers to the right in parentheses of branches indicate the GenBank accession number. Roman numerals denote the different genotypes of DENV-3.

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Vancomycin-Resistant Enterococci Outbreak, Germany, and Calculation of Outbreak Start

Ulrich Sagel,* Berit Schulte,† Peter Heeg,† and Stefan Borgmann†‡

On the basis of a large outbreak of vancomycin-resistant *Enterococcus faecium* in a German university hospital, we estimated costs (≈1 million Euros) that could have been avoided by early detection of the imminent outbreak. For this purpose, we demonstrate an easy-to-use statistical method.

Recently, vancomycin-resistant *Enterococcus faecium* (VRE) has been detected with increasing frequency in Germany (*1*). Although some hospitals have reported only sporadic findings, others have been faced with extended outbreaks (*2–5*). Apart from threatening patient health, these pathogens have an unfavorable economic impact on resources for healthcare in general. Control of VRE has proven to be costly and time-consuming (*6*). We therefore examined to what extent early implementation of control measures could have prevented these undesirable consequences in an outbreak of VRE at a university hospital in southwestern Germany.

At this hospital ≈68,000 inpatients and 220,000 outpatients are treated each year. In 2003, 5 VRE-colonized patients were identified. In 2004, VRE were first detected in April. While at most 3 cases per month were observed through July, 8 colonized patients were found in August (Figure 1, panel A). The number of colonized patients remained relatively high in the following months. By the end of December, the cumulative number of patients with VRE was 48 (Figure 1, panel B). Although medical microbiologists were concerned about a possible outbreak as early as August, decision makers were reluctant to acknowledge a situation that needed action to be taken until January 2005. At that time, an infection control program was implemented. It included VRE screening in stool and anal/rectal swab samples from patients exhibiting an increased risk for VRE carriage (*2*). This program resulted in a sharp increase of detected cases to a total of 105 patients for February and March 2005 (Figure 1, panel A).

Retrospectively, we examined whether comprehensible proof of an imminent outbreak could have been given early enough to have convinced decision makers of the need for control measures. For early outbreak detection, we have chosen a simple approach that can be easily adopted by infection control nurses dealing with multiresistant patho-

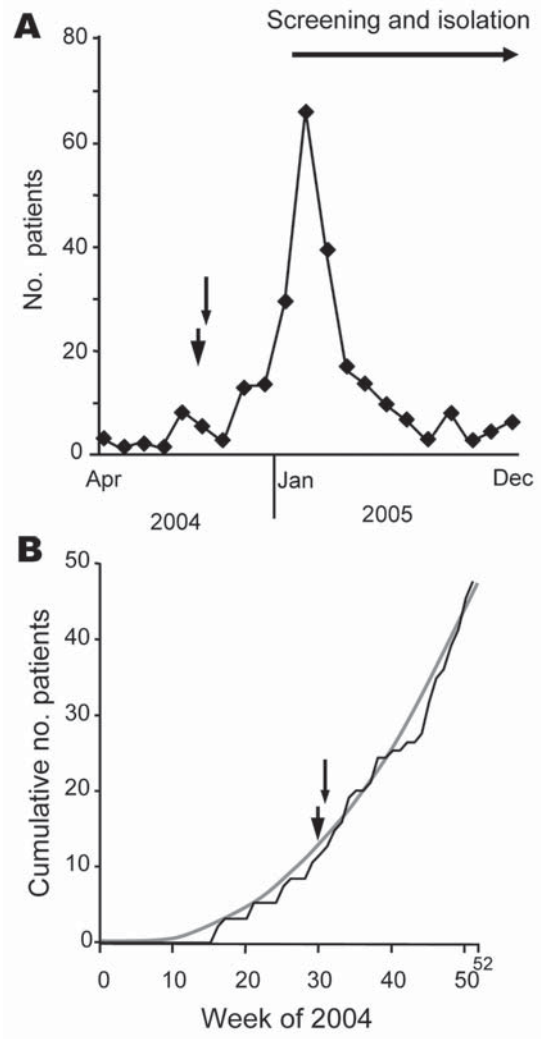

Figure 1. Course of vancomycin-resistant enterococci (VRE) outbreak at a German university hospital and time point (arrowhead, 30th calendar week; arrow, 31st calendar week) when outbreak alert could have been given. A) Number of VRE-carrying patients treated in a university hospital in 2004 and 2005. Given is the number of patients who were identified for the first time within a certain month (incident cases). In 2004 the first VRE patient was discovered in April 2005. B) Sum of VRE-exhibiting patients (cumulative number of patients [incident cases]) within distinct calendar weeks in 2004 (black line). Trend line (gray line) indicates exponential increase of numbers of incident cases ($y = 0.002$, $\chi^2 - 0.3497 \times 1.0299$ [$R^2 = 0.9918$]).

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gens usually occurring only sporadically (*7*). It is based on a Poisson distribution that describes the probability of rare, independent events in determined frames such as equal time periods (*8*). The course of a Poisson distribution is described by a complex formula containing several constants and 1 variable. This variable may be the number of VRE incidents that had happened in an earlier period (reference period). Knowing this, one can estimate the smallest number above a reference number to occur with a probability of $p<0.05$. Thus, for any small reference number, one can calculate a threshold number that cannot be explained by chance (respectively, a probability of $p<0.05$) and therefore suggests an imminent outbreak (Figure 2).

Sporadic cases of peculiar multiresistant pathogens at a hospital within a calendar year would be such a rare event. In 2003, 5 cases of VRE were observed within 1 hospital; therefore, one would expect some number of cases close to 5 to appear in 2004 by chance. As shown in Figure 2, if one considers 5 cases as the reference number, the occurrence of 10 cases would justify an outbreak alert. If one accounts for some underreporting in the reference period and assumes 7 cases as reference, 12 cases would be the threshold. In this example, the 10th VRE case in 2004 appeared at the 30th calendar week, and the 12th case at the 31st calendar week.

Using this method, one must be aware of some limitations. Diagnostic awareness and procedures should not have changed within the compared periods. Furthermore, comparing calendar years, as shown in our example for ease, may lead to late alerts; we should have taken the first 30 (or 31) weeks of 2003 as a more appropriate reference period.

Two unusual aspects of our example deserve mention: First, the outbreak was caused by 2 strains (ST203, ST280) and not by 1 strain. While strain ST203 was found in various departments, ST280 VRE was isolated from patients in 2 adjacent intensive care units (*2*). Apparently, this did not jeopardize the usefulness of the outbreak alert method we used. Second, other hospitals in the region also had reports about clusters of VRE (*5*). Therefore, hygienic measures may also have been hampered by admission of some colonized patients from outside the hospital (*9*). Regardless of whether cases were caused by within-hospital transmission or by introduction from outside, a markedly increased number of cases requires attention and control measures to prevent further spread.

On the basis of our calculations, outbreak control measures could have been justified by a comprehensible statistical method in August 2004 (30th/31st calendar week). Instead, the cumulative numbers of cases increased exponentially and insidiously for 4 more months until the decision makers accepted the need for an infection control program (Figure 1, panel B). Fortunately, hygienic measures

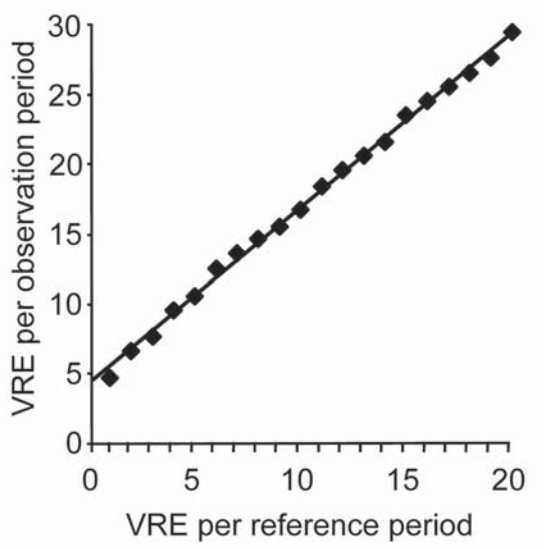

Figure 2. Alert threshold ($p<0.05$) derived from Poisson distribution; alert number for affected patients within an observation period depends on the number of patients found in a reference period. VRE, vancomycin-resistant enterococci.

effectively reduced the numbers of incident cases (Figure 1, panel A). Nonetheless, the impact of dozens of cases was high, and it took strong efforts and several months to get the outbreak under control (i.e., only a few cases of VRE strain ST203 still prevalent in the hospital).

Complete calculation of costs compared with those that could have been prevented by an early outbreak alert is complicated and retrospectively hard to achieve. For a crude estimation of minimum of avoidable costs, we assume that taking measures as early as August 2004 would have led to controlling the outbreak by January 2005. Since control measures in 2005 have sharply reduced the number of incident cases to a low level (Figure 1, panel A), admission from outside appears to play a minor role.

Therefore, we examined VRE patients identified from February through March 2005 (n = 105). Conclusive data were obtained from 93 patients who were kept in isolation for a total of 2,631 days. In the hospital, only 2 rooms were available for individual patient isolation. Per isolation day, there would have been a loss of income of ≈615 Euros for unused beds due to rooms occupied for isolation measures. This would have added 1,618,065 Euros in a completely occupied hospital. However, in 2004 and also in 2005, the occupancy of the hospital was only ≈80%, which lowered the loss of income by 40%. With total occupancy, the loss of income would be reduced to 970,839 Euros. However, because total occupancy will not be achieved in practice, the real loss of income was some amount between 970,839 Euros and 1,618,065 Euros. It is sufficient to calculate the severe loss of income to illustrate that the economic effects

of a large outbreak of VRE in a hospital can reach a loss of ≈1 million Euros. In a recent analysis of a VRE outbreak in Australia that resulted in the colonization of 64 patients, the costs were calculated at 2.7 million Australian dollars (*6*).

From our experience, we recommend making use of easy-to-use statistical models to detect and prevent imminent outbreaks as soon as possible. In this way, costs that may easily exceed 1 million Euros can be prevented.

Dr Sagel is a physician and microbiologist who has worked at 4 different microbiology laboratories in Germany. His main research interest is the epidemiology of antimicrobial drug–resistant bacteria in hospitals.

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Plasmodium falciparum Malaria and Atovaquone-Proguanil Treatment Failure

Rémy Durand,*†‡ Virginie Prendki,* Johann Cailhol,* Véronique Hubert,‡ Pascal Ralaimazava,* Laurent Massias,‡ Olivier Bouchaud,* and Jacques Le Bras*‡§

We noticed overrepresentation of atovaquone-proguanil therapeutic failures among *Plasmodium falciparum*–infected travelers weighing >100 kg. We report here 1 of these cases, which was not due to resistant parasites or impaired drug bioavailability. The follow-up of such patients should be strengthened.

Fewer than 25 cases of falciparum malaria that failed to respond to atovaquone-proguanil (A-P) have been noted in published articles since the 1996 registration of Malarone (GlaxoSmithKline, Marly-le-Roi, France) (*1*,*2*). Well-documented cases that were not attributed to suboptimal dosage or impaired bioavailability essentially due to vomiting, diarrhea, or both showed atovaquone-resistant parasites in the recrudescent isolate, with Y268S or Y268N cytochrome b mutations (*2*–*10*). We report the case of treatment failure that was not due to resistant parasites or impaired drug bioavailability.

The Case

During February 2007, a 39-year-old man who was born in Africa but lived in France since 1996 traveled to Kinshasa, Democratic Republic of Congo, for a 1-month vacation in which he visited friends and relatives. He was 6 feet tall with strong musculature and weighed 115 kg (body mass index = 34.3). He did not use chemoprophylaxis and did not take antimalarial self-treatment before seeking medical advice. Two days after returning to France, a fever and other signs of uncomplicated malaria attack developed (chills, arthralgia, asthenia) without vomiting. Three days after onset of symptoms, the patient sought care at the Avicenne Hospital, Bobigny, France. Blood smears performed at admission showed 1.6% *Plasmodium falciparum* parasitemia. The patient was hospitalized and immediately treated with the standard dosage of A-P (Malarone, 4 tablets each day for 3 days given with the main meal; each tablet contains 250 mg of atovaquone and 100 mg of proguanil hydrochloride). The patient experienced no vomiting or diarrhea. His fever abated by day 3 of treatment, and malaria smears at that time showed 0.07% of morphologically altered parasites (these figures are not uncommon because A-P is known to act relatively slowly). The patient was discharged on day 3. The patient did not return until his scheduled appointments for control of parasitemia on days 7 and 28. The patient was apyretic, and parasitemia was negative by day 7 on thin and thick blood smears. On day 28, the patient was apyretic, but thin and thick smears showed *P. falciparum* trophozoites (0.001% parasitemia on thin smear and 16 trophozoites per 1,000 leukocytes on thick smear). The patient was then successfully retreated with 650 mg quinine base orally 3× daily for 7 days.

Day 0 in vitro phenotype showed parasite susceptibility to atovaquone, with a 50% inhibitory concentration (IC_{50}) value of 10 nmol/L (in vitro resistance threshold >40 nmol/L [*11*]). Day 28 in vitro susceptibility was not assayed because of insufficient parasite density. DNA sequencing showed that both day 0 and day 28 isolates had wild-type sequence of cytochrome b. Genotyping of the *pfdhfr* gene showed that the 3 major *pfdhfr* mutations (at position 51, 59, and 108) associated with cycloguanil resistance were found in isolates from day 0 and day 28. The number and the proportions of genotypes within isolates were determined by a fragment analysis method based on the polymorphism of the gene encoding merozoite surface protein-2 (*12*). Day 0 and day 28 isolates contained the same majority genotype, with the 727-bp msp-2 allele representing >80% of isolates. This parasite population analysis did not show the selection of a minority-resistant genotype by A-P treatment. These results did not show either the emergence of mutant codon 268 cytochrome b within the 727-bp msp-2 dominant genotype. High-performance liquid chromatography on day 3 of treatment (performed 20 h after the last drug intake) showed an atovaquone plasma concentration of 3.1 μg/mL. High interpatient variability has been reported, but this value showed initial adequate drug concentration and excluded impaired bioavailability (*13*).

Thus, this patient, who had correctly taken A-P tablets with food and did not vomit, showed correct plasma drug concentration on day 3 but did not show clearance of A-P–susceptible parasites on day 28, although he was asymptomatic. Reinfection was excluded because the patient was treated after returning to France (which is a non–malaria-endemic area). All these data suggest that the standard drug regimen led to suboptimal dosage in this patient. Apparently correct initial atovaquone concentration on day 3 did not predict the outcome of treatment because A-P acts slowly, and most reported A-P therapeutic failures were late failures. Drug interactions that could have lowered A-P

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plasmatic concentration were excluded because, other than A-P, the patient received only acetaminophen.

Conclusions

The most plausible cause of this late therapeutic failure is the relatively insufficient dosage due to increased oral clearance and volume of distribution of atovaquone in this patient who weighed ≥100 kg. According to the relationships between oral clearance of atovaquone and weight, and between volume of distribution and weight, these parameters increase by 40% in comparison to those in a patient of 70 kg (*13*). The effect may be less marked for proguanil because, unlike atovaquone, it becomes concentrated in the erythrocytes. However, proguanil likely does not act by itself in A-P association but only facilitates the atovaquone activity (*14*). Likewise, the observed *pfdhfr* triple mutant likely had no effect on the intrinsic activity of proguanil but only reflected the high frequency of this haplotype in West Africa.

Another hypothesis is that the treatment failure was due to resistant parasites not related to the cytochrome b mutations. Some rare therapeutic failures remain unexplained by phenotypic or genotypic data. Such a parasite should have emerged during treatment, because the IC_{50} of the day 0 isolate was susceptible. We know of only 1 other reported case of A-P treatment failure not associated with cytochrome b mutations and not related to incorrect dosage or impaired bioavailability (*15*). In the case reported by Wichmann et al. (*15*), clonal analysis of pre- and posttreatment isolates was not performed, which limited the interpretation of DNA sequencing; the weight of the patient was not mentioned.

Most previously reported A-P therapeutic failures were late failures: patients sought care or parasitemia was detected >3 weeks after first day of treatment in 13 of 21 cases (*2–5,7–9,15*). These data underline the usefulness of the day 28 appointment in detecting late A-P therapeutic failures. In addition, this appointment may provide the opportunity to detect asymptomatic parasitemia (*2*), as occurred in the present case.

We noticed an overrepresentation of A-P therapeutic failures among patients who weighed ≥100 kg. In a series of 347 *P. falciparum*–infected travelers, 3 of 12 patients who weighed 100–115 kg exhibited therapeutic failure while receiving the standard A-P regimen (only 2 therapeutic failures were reported among the other 335 patients who weighed <100 kg). Two of these patients >100 kg showed mutant parasites on the date treatment failure was recognized (*2*); the third treatment failure (the present one) was due to susceptible parasites. Thus, relative suboptimal dosage of the standard A-P regimen in patients >100 kg led to either failure to control susceptible parasites or emergence of resistant ones. DNA point mutations conferring atovaquone resistance may emerge more easily in patient who have received suboptimal dosage. However, not all patients weighing ≥100 kg seen in our center had A-P therapeutic failures. Eleven of these 12 patients were from Africa and could have possessed residual immunity, which could have helped them clear parasites.

A-P is safe and therapeutic failure remains rare. Nevertheless, the overrepresentation of failures in patients >100 kg argues for strengthening the follow-up monitoring of those patients. Moreover, the weight of a patient could be taken into account in the dosage of A-P prescribed for similar cases in the future.

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Prolonged *Plasmodium falciparum* Infection in Immigrants, Paris

Eric D'Ortenzio,*[1] Nadine Godineau,† Arnaud Fontanet,‡ Sandrine Houze,*§ Olivier Bouchaud,¶# Sophie Matheron,*, and Jacques Le Bras*§**

Few immigrant travelers have *Plasmodium falciparum* infections >2 months after leaving malaria-endemic areas. We conducted a case–control study to identify factors associated with prolonged *P. falciparum* infection in immigrant travelers. Results suggest that *P. falciparum* infection should be systematically suspected, even months after travel, especially in pregnant women and first-arrival immigrants.

Approximately 100 countries endemic for malaria are visited by 125 million international travelers yearly, and >30,000 contract imported malaria (*1*). In France, the number of imported cases of *Plasmodium falciparum* malaria was estimated to be 4,500 in 2004, with a median time of 10 days between departure from an area endemic for malaria and diagnosis (*2*). The duration of a *P. falciparum* infection in humans is generally believed not to exceed 12 months. Most epidemiologic studies show that few patients have malaria onset >2 months after returning from travel (*3*,*4*). Late occurrence of infection could have severe consequences if physicians do not relate symptoms suggestive of malaria to travel history. Another risk is transfusion-transmitted malaria from an asymptomatic carrier of *P. falciparum* trophozoites (*5*).

Cases of late occurrence of *P. falciparum* malaria have been reported (*6*–*9*), but risk factors are unknown. The objective of this study was to determine the incidence and identify factors associated with prolonged *P. falciparum* infection in immigrant travelers.

The Study

A case–control study was conducted among patients with *P. falciparum* malaria diagnosed at Bichat-Claude Bernard and Saint-Denis Hospitals in Paris, France. Many African immigrants come to these hospitals. Participants traveled to or lived in an area endemic for malaria and had a *P. falciparum* infection during 1996–2005. The diagnostic criterion was *P. falciparum* trophozoites on a blood smear confirmed by the Centre National de Reference du Paludisme (CNRP) in Paris, without epidemiologic evidence of autochthonous, transfusion-transmitted, or occupational malaria. Case-patients had *P. falciparum* infections detected >59 days after their arrival in France. Controls had *P. falciparum* infections detected ≤30 days after their arrival. For each case-patient, 4 controls were matched by calendar year and hospital of diagnosis (70 cases and 280 controls). Data were collected from the CNRP database in which all cases are prospectively included and medical records are checked. We only considered immigrants (persons born in an area endemic for malaria and residing in France), which resulted in 61 case-patients and 197 controls. We distinguished first-arrival immigrants (persons who emigrated to France and never returned to areas endemic for malaria) from visiting friends and immigrant relatives (persons who traveled back to areas endemic for malaria after immigration to France).

Logistic regression was used to identify factors associated with prolonged *P. falciparum* infection and estimate odds ratios (ORs) and 95% confidence intervals (CIs). For multivariate analysis, variables with p values <0.25 were introduced into the model and removed after a backward stepwise approach, which resulted in only values with $p<0.05$ in the final model (except for age groups). Statistical analysis was performed by using Stata software version 8.2 (Stata Corporation, College Station, TX, USA).

During the 10-year period, 61 (2.3%) late infections occurred among 2,680 diagnosed *P. falciparum* malaria infections. The median diagnosis delay was 5 months (interquartile range 3–9 months). These infections included 10 patients (5 pregnant women, 2 HIV-positive patients, and 3 first-arrival immigrants) with clinical malaria >1 year after their arrival. Four of them, all pregnant women, had clinical malaria >3 years after their arrival. For the case–control study, 197 controls were compared with 61 case-patients (Figure). Table 1 shows the main characteristics of case-patients and controls. Case-patients were younger (median age 30.6 years vs. 34.5 years, $p = 0.04$) and more often female (54.1% vs. 38.1%, $p = 0.03$) than controls. The mean parasitemia level was lower for case-patients than for controls (0.6% vs. 1.4%, $p = 0.04$), including patients with 8 asymptomatic cases versus none of the controls (in these cases, diagnosis of malaria was made through systematic checking).

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Among immigrant travelers, 3 groups had a higher risk for prolonged *P. falciparum* infection: pregnant women, first-arrival immigrants, and HIV-positive patients. A total of 27.9% (n = 17) of the patients were pregnant women, with a median (range) age of 22 (16–36) years. All were of African origin and had become pregnant in France; 10 (58.8%) were in their second trimester, 5 (29.4%) were in their third trimester. First-arrival immigrants were younger than other patients (mean age 26.2 vs. 37.6 years, p = 0.001). All patients were of African origin except for 1 Indian man.

Table 1. Univariate analysis of factors associated with prolonged *Plasmodium falciparum* infection in 248 immigrant travelers*

Variable	Case-patients (n = 61), no. (%)	Controls (n = 197), no. (%)	OR (95% CI)	p value
Sex				
Female	33 (54.1)	75 (38.1)	1	
Male	28 (45.9)	122 (61.9)	0.52 (0.29–0.93)	0.03
Age, y				
<5	2 (3.3)	10 (5.1)	1	
5–14	5 (8.2)	18 (9.1)	1.39 (0.22–8.51)	
15–60	53 (86.9)	163 (82.7)	1.63 (0.34–7.66)	0.83
>60	1 (1.6)	6 (3.1)	0.83 (0.06–11.23)	
Origin				
Sub-Saharan Africa	55 (90.2)	181 (91.9)	1	
Comoros Islands	5 (8.2)	14 (7.1)	1.18 (0.41–3.41)	
Other	1 (1.6)	2 (1)	1.65 (0.15–18.5)	0.89
South America	0	1 (0.5)	NA	
Caribbean	0	1 (0.5)	NA	
India	1 (1.6)	0	NA	
First-arrival immigrant				
No	24 (39.3)	183 (92.9)	1	
Yes	37 (60.7)	14 (7.1)	20.15 (9.54–42.57)	<0.001
Region of malaria acquisition				
West Africa	32 (52.5)	132 (67)	1	
Central Africa	22 (36.1)	44 (22.3)	2.06 (1.09–3.92)	0.03
East Africa	1 (1.6)	2 (1)	2.06 (0.18–23.46)	
Comoros Islands	5 (8.2)	18 (9.1)	1.15 (0.4–3.32)	
Other	1 (1.6)	1 (0.5)	4.12 (0.25–67.74)	
Chemoprophylaxis				
No	50 (82)	118 (59.9)	1	
Yes	9 (14.8)	71 (36)	0.3 (0.14–0.65)	0.002
Unknown	2 (3.2)	8 (4.1)	NA	
Prophylaxis with mefloquine				
No	56 (91.8)	186 (94.4)	1	
Yes	3 (4.9)	3 (1.5)	3.32 (0.65–16.92)	0.15
Unknown	2 (3.3)	8 (4.1)	NA	
Antimalarial self-medication				
No	55 (90.2)	171 (86.8)	1	
Yes	5 (8.2)	15 (7.6)	1.04 (0.36–2.98)	0.95
Unknown	1 (1.6)	11 (5.6)	NA	
Men	28 (45.9)	122 (61.9)	1	
Nonpregnant women	16 (26.2)	69 (35)	1.01 (0.51–2)	
Pregnant women	17 (27.9)	6 (3.1)	12.35 (4.46–34.14)	<0.001
HIV status				
Negative	22 (36.1)	42 (21.3)	1	
Positive	12 (19.7)	6 (3.1)	3.82 (1.26–11.56)	0.02
Unknown	27 (44.3)	149 (75.6)	NA	
Symptomatic malaria				
No	8 (13.1)	0 (0)	1	
Yes	53 (86.9)	197 (100)	0.3 (0.25–0.47)	<0.001
Parasitemia†				
High	2 (3.3)	18 (9.1)	1	
Low	59 (96.7)	179 (90.9)	1.61 (0.53–4.9)	0.4

*OR, odds ratio; CI, confidence interval; NA, not applicable.
†Parasitemia (parasitized erythrocytes) was considered high if ≥4% and low if <4% by World Health Organization criteria.

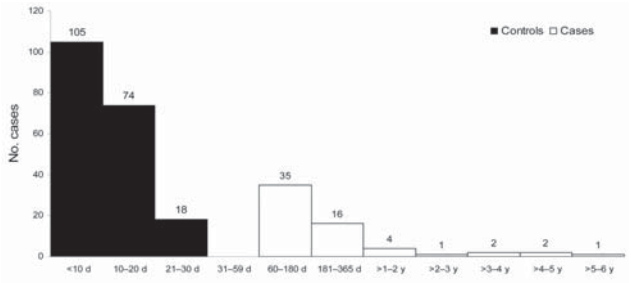

Figure. Delay in days or years between arrival in France and diagnosis of imported *Plasmodium falciparum* malaria, Bichat-Claude Bernard Hospital and Saint Denis Hospital, Paris, France, 1996–2005.

HIV infection was associated with prolonged infection, but HIV status was not introduced into the final model because of missing data. Although chemoprophylaxis with chloroquine-proguanil was less common among case-patients than controls (8.5% vs. 21.2%, p = 0.03), the reverse was seen, although not significantly, with mefloquine use (4.9% vs. 1.5%, p = 0.15). Multivariate analysis (Table 2) showed that factors positively and independently associated with prolonged *P. falciparum* infection in immigrant travelers were being a first-arrival immigrant (OR 22.93, 95% CI 9.74–53.96, p<0.001), being a pregnant woman (OR 4.21, 95% CI 1.13–15.77, p = 0.03), and mefloquine prophylaxis (OR 11.55, 95% CI 2.06–64.78, p<0.005).

We also observed cases of malaria in a 26-year-old Caucasian man and a 2-year-old African girl who were hospitalized with diagnosis delays of 221 days and 127 days, respectively. The man was a French expatriate who lived in Madagascar for 2 years and took regular chloroquine-proguanil prophylaxis. He was hospitalized 7 months after his return with severe *P. falciparum* malaria (impaired consciousness) and responded to treatment. The girl had traveled in Mali for 2 weeks and took regular chloroquine-proguanil prophylaxis. She was hospitalized 4 months after her return with uncomplicated *P. falciparum* malaria occurring concomitantly with a *Salmonella* spp. infection that had been treated 1 week earlier with ceftriaxone.

Table 2. Factors independently associated with prolonged *Plasmodium falciparum* infection in 248 immigrant travelers*

Variable	OR (95% CI)*	p value
Age, y		
<5	1	
5–14	1.45 (0.15–13.74)	
15–60	1.72 (0.25–12)	
>60	3.04 (0.16–56.25)	0.45
First-arrival immigrant		
No	1	
Yes	22.93 (9.74–53.96)	<0.001
Men	1	
Nonpregnant women	0.67 (0.28–1.59)	
Pregnant women	4.21 (1.13–15.77)	0.03
Use mefloquine		
No	1	
Yes	11.55 (2.06–64.78)	0.005

*OR, odds ratio; CI, confidence interval.

Conclusions

Three independent factors were positively associated with prolonged *P. falciparum* infection: being a first-arrival immigrant, being a pregnant woman, and taking mefloquine prophylaxis. The first 2 factors most likely reflect partial control of parasitemia by acquired immunity. Persons living in areas with high transmission of malaria acquire this immunity during childhood. In these areas, *P. falciparum* infections in adults are mostly asymptomatic with transient low parasitemia levels (*10*). Chronic asymptomatic carriage of *P. falciparum* helps prevent symptomatic malaria attacks (*11*). In a previous study, 29% of asymptomatic Liberian children had detectable *P. falciparum* 4 weeks after immigration to the United States (Minnesota) (*12*). We postulate that many first-arrival immigrants are asymptomatic *P. falciparum* carriers upon their arrival in France. Their immunity probably prevents clinical symptoms for a few months, but in the absence of reinfections their immunity would decrease and symptoms would occur. In some cases, *P. falciparum* infections may not be the cause of illness when patients come to a hospital.

Several immunologic mechanisms have been suggested to explain late manifestations of *P. falciparum* malaria in pregnant women. A decrease in immunity during pregnancy could be one explanation (*10*). Other authors have suggested that antigenic variability could be responsible for impaired control of parasitemia (*13*). The role of mefloquine prophylaxis in delayed onset of malaria has been suggested (*14*). We found a positive association between mefloquine use and prolonged *P. falciparum* infection, but this drug was seldom used by our study group. This association is probably caused by the long half-life of mefloquine (>3 weeks).

This study also highlights the risk for blood transfusion–transmitted malaria, a rare but serious complication. Mungai et al. (*15*) reported 32 cases of transfusion-transmitted *P. falciparum* malaria in the United States during 1963–1999 (mortality rate 18.8%). Current US guidelines recommend obtaining a thorough travel history and deferring blood donation if potential donors have emigrated from areas endemic for malaria in the preceding 3 years. However, this measure may not prevent transmission if *P. falciparum* is present for >3 years (as in 4 pregnant women in our study). In France, systematic serologic analysis for *Plasmodium* spp. in blood donors born in areas endemic for malaria was implemented in 2002 (*5*).

Our findings suggest that physicians should consider the risk for prolonged *P. falciparum* infection in immigrant

pregnant women and first-arrival immigrants even without recent travel to a country endemic for malaria. The prevalence of asymptomatic *P. falciparum* carriers in France or other northern countries is unknown but could be high with the increase in immigration. Public health authorities should be aware of the risk these persons represent for blood donations.

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etymologia

Candida

[kan′-di-də], from the Latin—candidus (glowing white)

A genus of yeastlike Fungi Imperfecti (for which no sexual reproductive stage is known) of the family *Cryptococcaceae* that produce yeast cells, mycelia, pseudomycelia, and blastospores. When grown in the laboratory, *Candida* appears as large, round, white or cream colonies on agar plates. *C. albicans* infection (or thrush) features distinctive white mouth lesions; "albicans" means becoming white. *C. dubliniensis*, first identified in 1995 at the University of Dublin, is an opportunistic pathogen that can cause both superficial and invasive infections, particularly in the immunocompromised.

Source: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; Sullivan, DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV-infected individuals. Microbiology 1995;141:1507–121.

Candida dubliniensis Meningitis as Delayed Sequela of Treated *C. dubliniensis* Fungemia

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We present a case of *Candida dubliniensis* meningitis that developed 2 months after apparently successful treatment of an episode of *C. dubliniensis* candidemia in a heart-lung transplant recipient in Australia. This case highlights the importance of follow-up in patients with candidemia or disseminated infection, especially in immunosuppressed patients.

The patient, a 48-year-old man, was admitted to St Vincent's Hospital, Sydney, Australia, in February 2007 for a heart and bilateral lung transplant for a familial dilated cardiomyopathy with severe secondary pulmonary hypertension. The operation was uneventful. Postoperatively, the patient was admitted to the intensive care unit (ICU), and immune-suppressive agents (cyclosporine, methylprednisolone, azathioprine) and prophylaxis against opportunistic infections (ganciclovir, cotrimoxazole, nebulized amphotericin B at 10 mg twice a day) were begun. After an initial loading dose of 70 mg caspofungin, 50 mg daily was continued for treatment of infection with a *Candida* spp. isolated from a blood culture on postoperative day 9. Standard phenotypic methods and API 32C (bioMérieux, Marcy l'Etoile, France) confirmed a *C. dubliniensis* with 99% probability. The same organism was grown from pleural fluid (empyema) and urine (candiduria, normal renal imaging).

Although the isolate's fluconazole MIC was 0.025 μg/mL (YeastOneYO8, TREK Diagnostic Systems, Ltd., East Grinstead, UK), caspofungin (MIC 0.06 μg/mL) was administered because of ongoing dialysis-dependent renal impairment and abnormal liver function test results related to hepatic ischemia. The transesophageal echocardiogram results were normal. Ophthalmology review failed to demonstrate endophthalmitis. Candidemia clearance was confirmed by negative blood cultures (days 7, 8, and 10 of caspofungin). Repeat urine cultures were sterile. A chest and abdominal computed tomography scan (on day 32 of antifungal therapy) showed a normal liver, spleen, and renal tract as well as bilateral reaccumulated pleural effusions after removal of the intercostal chest drains. However, microbiologic cure was confirmed by a repeat pleural aspiration. In addition, chest radiographs before discharge confirmed total resolution of the pleural effusion. HIV serologic test results were negative. Dose adjustments for cyclosporine and prednisolone were the only changes instituted to the immune-suppressive agents during the patient's illness. The oral prednisolone was slowly tapered from an initial 50 mg/day to a maintenance dose of 10 mg/day in March. The patient was discharged from intensive care on day 14 and from hospital 52 days posttransplant, in April 2007; he was not receiving any antifungal agents on discharge. The total duration of therapy for disseminated candidiasis was 40 days, consisting of caspofungin (28 days) followed by fluconazole (400 mg/day for 12 days).

The patient was seen 2 months later, in June 2007, with a 3-week history of progressive headache, early morning nausea, vomiting, and weight loss. There was no history of fevers or rigors. Examination showed no neck stiffness, photophobia, or focal neurologic signs. A magnetic resonance imaging scan showed enhancing meninges consistent with meningitis. A lumbar puncture yielded clear cerebrospinal fluid (CSF) with 116 x 10^6 leukocytes/L (79% neutrophils and 14% lymphocytes), an elevated protein level of 1,224 mg/L (normal range 0–400 mg/L), and a reduced glucose level of 1.7mmol/L (normal range 2–4 mmol/L) with a concurrent serum glucose level of 5.5 mmol/L(normal range 3.0–7.8 mmol/L). No fungi were seen on Gram stain. At 24 hours, the primary plates and broth culture grew a budding yeast that was identified with a 99% probability as *C. dubliniensis* on API 32C (bioMérieux).

Molecular confirmation was performed. Genomic DNA was extracted from the culture by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). *C. dubliniensis*–specific PCR and internal transcribed spacer (ITS) regions of the rRNA gene complex were amplified as previously described (*1*,*2*). The PCR products were purified for sequencing by using the QIAquick PCR Purification Kit (QIAGEN). The sequences were compared to those available in the GenBank databases by using the BLASTN program (www.ncbi.nlm.nih.gov/BLAST). The ITS gene sequences generated showed a 100% similarity to strains of *C. dubliniensis* (GenBank accession nos. DQ 355947, AF405231, AJ865083, AJ865082, AJ865081). Unfortunately, the initial blood culture isolate was no longer available for comparative sequencing. However, the API 32C and susceptibility profiles (YeastOne YO8) were identical, a finding that suggested that the 2 strains were identical.

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Several blood cultures and urine cultures (when the patient was not receiving antifungal therapy) were negative. A chest radiograph was clear with no evidence of effusion. A transthoracic echocardiogram and ophthalmology review were clear for signs of metastatic candidiasis. Fluconazole therapy was begun, and the patient made a full recovery.

Candida is an important pathogen in critically ill patients. Yeasts account for 8%–10% of nosocomial blood culture isolates with an increased incidence in immune-suppressed patients (*3*). Multiple species cause candidemia; however, 5 species—*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei*—account for >95% of all cases worldwide, including Australia (*3*,*4*).

C. dubliniensis shares phenotypic characteristics with *C. albicans* on routine laboratory testing and therefore was only recognized as a novel species with the advent of molecular testing. It remains an uncommon isolate, accounting for <2% of all candidemias (*4*). The original reports of *C. dubliniensis* were in mucosal disease HIV-infected patients and patients not infected with HIV (*5*). Subsequent candidemia was reported from Europe, North America, and Australia in a wide variety of patients with multiple serious medical problems (*6*–*9*). *C. dubliniensis* candidemia in solid organ transplant recipients is rare (*10*).

Meningitis is a rare manifestation of disseminated disease. Risk factors for meningitis are similar to those associated with invasive candidiasis (*4*). The risk of developing this complication is unknown. However, 2 specific patients groups, premature neonates and neurosurgical patients, are at increased risk (*11*,*12*). *C. albicans* accounts for 70%–100% of all meningitis isolates. Other reported species include *C. glabrata, C. tropicalis, C. parapsilopsis,* and *C. lusitaniae* (*4*). Our patient represents, to our knowledge, the first documented case of *C. dubliniensis* meningitis.

Symptoms of fungal meningitis include fever, headache, altered mental status, and meningism. Focal neurologic signs are rare. The frequency and severity of symptoms vary between patient groups. In HIV-infected patients, fever (86%), headache (93%), and meningism (50%) occurred in most patients; by contrast, patients with neurosurgical devices had lower rates of meningism (18%) and headaches (18%) but comparable rates of fever (82%) (*10*). *Candida* meningitis in solid organ transplant recipients is extremely rare; symptoms are probably modified by the degree of immune-suppression, as illustrated in our patient.

The diagnosis of meningitis is established by a positive CSF culture. Multiple CSF specimens may be required. CSF parameters are variable, with a mild lymphocytic or polymorphonuclear pleocytosis and an increased protein level. Fungal elements are generally not seen. Thus, CSF abnormalities are indistinguishable from cryptococcal, tuberculous, and some bacterial meningitides (*13*).

Delayed complications occur after candidemia. Thus, consensus guidelines suggest 3 months' follow-up to detect these complications (*14*). Delayed meningeal infection following *C. albicans* candidemia has been documented; the meningitis occurred 3 months after "successful" therapy (*15*).

Our patient received curative therapy (negative repeat cultures) for disseminated candidiasis (candidemia, candiduria, and empyema) with caspofungin and fluconazole of adequate duration. Despite this treatment, our patient had delayed meningitis 2 months after therapy. Whether the meningitis was secondary to re-infection or reactivation of latent infection is unclear. Caspofungin was the cornerstone of therapy, and reactivation is possible with this antifungal agent because it has poor CSF penetration. However, re-infection cannot be excluded.

In conclusion, we present a case of delayed *C. dubliniensis* meningitis. This case highlights the need for clinicians to be aware of possible delayed complications despite apparently successful therapy. Furthermore, routine follow-up (at 3 months) should be considered for all patients following candidemia, especially immune-suppressed patients.

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Greek Goat Encephalitis Virus Strain Isolated from *Ixodes ricinus*, Greece

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A strain of Greek goat encephaltitis virus was isolated from engorged *Ixodes ricinus* ticks that had fed on goats in northern Greece. The strain was almost identical to the prototype strain isolated 35 years ago.

Tick-borne encephalitis (TBE) is a zoonotic infection of the central nervous system; it is transmitted by ticks from the family Ixodidae. In an ecologic sense the disease agent, TBE virus, is an arbovirus (arthropod-borne virus); taxonomically, it is a member of the genus *Flavivirus*, family *Flaviviridae.* According to the latest taxonomy on flaviviruses, TBE virus is a species in the mammalian tick-borne virus group and has 3 subtypes: European, Far Eastern, and Siberian (*1*). Louping ill virus belongs in the same mammalian group and has 4 subtypes: British, Irish, Spanish, and Turkish. It has recently been suggested that TBE and louping ill viruses belong in the same species (TBE virus), which has 4 types: western TBE virus, eastern TBE virus (which includes Far Eastern and Siberian subtypes), Turkish sheep encephalitis virus (which includes the Greek goat encephalitis [GGE] virus,) and louping ill virus (which includes Spanish, British, and Irish subtypes) (*2*).

Information about TBE and its epidemiology in Greece is limited. The first evidence of human infection with TBE virus was reported during an investigation of the etiology of the 1927–1928 dengue epidemic. In this investigation, antibodies to TBE virus were detected by hemagglutination and neutralization tests in 1 (1.8%) of 56 serum samples (*3*). Similar results (1.7%) were found in a survey of 1,128 serum samples (*4*). In March 1969, Vergina strain (the prototype strain of GGE virus) was isolated in Vergina village, northern Greece, from the brain of a newborn goat with encephalitis-like symptoms (*5*). It was suggested at that time that GGE virus might represent a third subtype because it differed antigenically from all strains belonging to the types I and II of TBE viruses that were known at that time and transmitted by *Ixodes persulcatus* and *I. ricinus*, respectively (*6*). Because *I. gibbosus* was the only *Ixodes* spp. tick found in the region of Vergina, it has been hypothesized that GGE virus is transmitted by this species.

A serologic study, which used the hemagglutination inhibition test, of animals living permanently in northern Greece showed that 16.8% of goats, 5.6% of pigs, 5.1% of sheep, 4.7% of horses, and 3.1% of cattle had antibodies to TBE virus (*7*). A seroepidemiologic survey conducted in various prefectures in Greece found that 1.7% of the population had antibodies to TBE virus (*8*). During a recent seroepidemiologic study conducted during 2003–2005 in northern Greece, highest prevalence (5.82%) of antibodies against TBE virus was observed in the Chalkidiki prefecture (*9*). In addition, a TBE case was serologically diagnosed in this area (*10*). We report isolation of a GGE virus strain from *I. ricinus* ticks collected in Vavdos village in the Chalkidiki prefecture.

The Study

From April through June and September through December, 2003–2006, a total of 703 adult Ixodidae ticks were collected from flocks of goats grazing permanently in 3 mountainous areas of Chalkidiki. Ticks were classified according to identification keys (Table) and grouped and assigned to pools of 10–15. Ticks were washed with sterile phosphate-buffered saline and homogenized in 500 μL of culture medium containing 4% fetal bovine serum and 500 IU/mL penicillin and streptomycin by the use of glass beads in a cell disrupter. The homogenized suspension was centrifuged at 2,500*g* for 5 min; 250 μL of the supernatant was used for RNA extraction by using TRIZOL LS Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and the rest was stored at –70°C until further use. PCRs were performed by using 2 different pairs of primers: 1 pair of degenerated primers for the 5′ end of the envelope (E) gene (*11*) and 1 pair from the C-terminal part of the nonstructural protein 5 (NS5) gene (*12*).

One pool of *I. ricinus* ticks was TBE-positive in both reverse transcription–PCR (RT-PCR) assays. The pool consisted of 1 female and 9 male ticks collected in November 2004 in Vavdos village. Ticks of all other species were negative. Assuming that 1 tick per pool was positive, the

Table. Species of ticks collected during 2004–2006, Chalkidiki, Greece

Tick species	Year			Total
	2004	2005	2006	
Ixodes ricinus	127	158	70	355
Rhipicephalus bursa	39	40	22	101
Rh. turanicus	25	0	0	25
Rh. sanguineus	76	0	0	76
Hyalomma marginatum	75	30	28	133
Boophilus annulatus	0	13	0	13
Total	342	241	120	703

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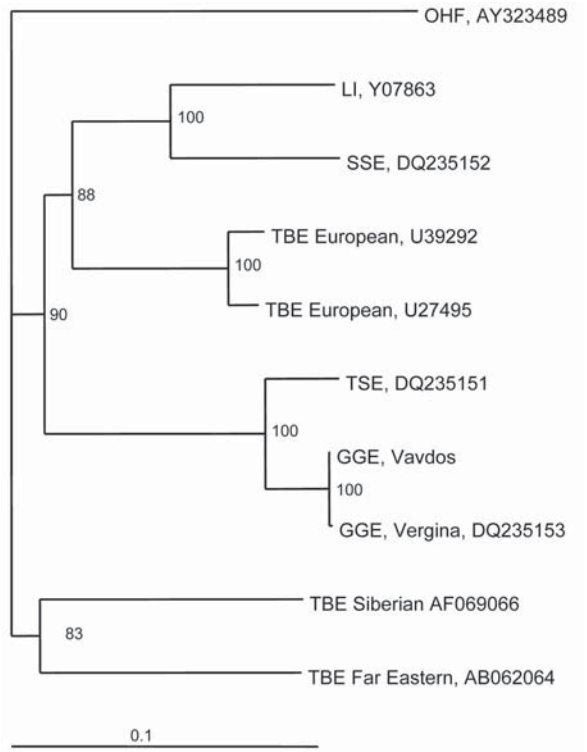

Figure 1. Phylogenetic tree of the envelope gene of tick-borne encephalitis (TBE) virus strains constructed by using PHYLIP software (http://evolution.genetics.washington.edu/phylip.html). Omsk hemorrhagic fever (OHF) virus was used as the out-group. The numbers at the nodes represent bootstrap values. LI, louping ill; SSE, Spanish sheep encephalitis; TSE, Turkish sheep encephalitis; GGE, Greek goat encephalitis. Scale bar, 10% nucleotide sequence divergence.

total frequency of infected *I. ricinus* ticks was 0.28%; annual tick infection rate was 0.78% for 2004.

Of the stored supernatant, 70 μL was inoculated onto Vero E6 cells; flasks were incubated at 37°C and passaged to fresh cells every 5 days. Viral supernatants were tested by immunofluorescent assay and RT-PCR. After the fifth consecutive passage, fluorescence was present and 3 more passages of the virus (Vavdos strain) were performed.

Nucleotide sequences of the viral genes encoding 1 NS protein (NS5) as well as the capsid (C), membrane (M), and E proteins were determined by using the above-mentioned and newly designed primers (GenBank accession nos. EF693938 and EF693939). A high degree of homology with the Vergina strain (DQ235153) was observed, although the 2 strains were isolated 35 years apart and the isolation sites were 140 km apart from each other. The 2 strains differed by 2 nucleotides (0.13%) in the E gene; 1 resulted in an amino acid change (aa 122, glutamic acid in Vavdos, glycine in Vergina), but they were identical in the C, M, and NS5 genes. Similar stable phylogenetic relationships were observed in TBE virus strains of other subtypes, which indicates that the virus is remarkably stable and not subject to major antigenic variation (*13*,*14*). A probable explanation might be that TBE virus evolves within a 2-host system; furthermore, tick-borne flaviviruses evolve at 0.56 times the rate of mosquito-borne flaviviruses because of the ticks' long life cycle and limited seasonal feeding activity (*15*).

In the phylogenetic tree based on the whole E gene (1,488 nt) of TBE viruses, Greek strains cluster together with Turkish sheep encephalitis virus (nt homology 95.5%) and form an independent clade with high bootstrap value, which might represent the southern subtype (Figure 1). Genetic distances of GGE strains and those of western, eastern, and louping ill subtypes are 16.3%, 20%, and 18.5%, respectively.

Vavdos is a mountainous village 800 m above sea level (23°26′31.1″E, 40°22′8.0″N). The area where goats were grazing was covered by typical Mediterranean low vegetation and was located at the edge of an oak forest (Figure 2). No signs of disease were present in any of the goats of the flock. In addition, the owner of the flock and his family did not report any TBE-like symptoms (but they refused to be tested for antibodies to TBE virus). The newborn goat from which the prototype GGE virus strain was isolated in 1969 had neurologic symptoms; in addition, many abortions had occurred in that flock. However, no virus was isolated from any other animal or ticks collected in northern Greece during that period, which suggests that TBE virus is rare.

Conclusions

Natural foci of GGE virus are present in northern Greece. The strain that circulates in Greece resembles that

Figure 2. Map of Greece showing location of Vergina and Vavdos villages.

isolated from sheep in Turkey, which has not yet been associated with disease in humans. Sequencing of the complete genome, including the more variable 3′-noncoding region, and neutralization tests that are in progress will give further insights into this group of viruses.

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Transmission of Hepatitis C Virus during Computed Tomography Scanning with Contrast

Helena Pañella,* Cristina Rius,* Joan A. Caylà,* and the Barcelona Hepatitis C Nosocomial Research Working Group[1]

Six cases of acute hepatitis C related to computed tomography scanning with contrast were identified in 3 hospitals. A patient with chronic hepatitis C had been subjected to the same procedure immediately before each patient in whom acute infection developed. Viral molecular analysis showed identity between isolates from patients with acute and chronic hepatitis C.

The most common mechanism involved in transmission of hepatitis C virus (HCV) is exposure to contaminated blood; sharing of syringes between drug users is currently the most frequent factor. However, in 40% of cases, the mechanism cannot be identified, and nosocomial transmission has an increasingly important role (*1*).

In recent years, transmission of HCV from an infected patient to a susceptible person during various healthcare-related procedures has been reported (*2*,*3*). The risk of transmission depends on the mechanism responsible, as well as on the prevalence of infected persons. Thus, in addition to use of shared vials for administration of heparin (*4*), physiological saline solution (*5*–*7*) or anesthetics (*8*,*9*), procedures carried out with contaminated equipment (*10*,*11*) or inappropriate practices of health personnel (*12*) have been proposed as mechanisms of nosocomial transmission. Molecular analysis of HCV has permitted comparison of different viral sequences to confirm transmission.

Hepatitis C infection is a mandatory reportable disease in Spain and is reported as a suspected infection as soon as it is clinically diagnosed. In Barcelona, after initial reporting of a case of hepatitis C, an epidemiologic survey is conducted by trained personnel from the epidemiologic service to identify possible sources of infection (sexual or household contact with an HCV case, use of multidose medications, transfusions, surgical interventions or other invasive procedures during their hospital stay) and to implement appropriate control measures. The aim of the present study was to describe several cases of nosocomial HCV transmission affecting patients in which the identified possible source of transmission was a computed tomography (CT) scan with contrast in different hospitals in Barcelona in 2004.

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The Study

From August through November 2004, 6 cases of acute hepatitis C associated with a CT scan with contrast were diagnosed in 2 public hospitals and 1 private diagnostic center. Only 1 was detected by active case finding. Three cases were women and 3 were men (ages 29, 47, 55, 57, 58 and 61 years, respectively). All but 1 had symptoms compatible with acute hepatitis with dates of onset from July 8 to September 18, 2004. All had increased serum transaminase levels, positive serologic results for hepatitis C, and HCV RNA detected by reverse transcription–PCR. All cases were investigated and other sources of transmission were ruled out; all participants were outpatients with appointments and did not undergo any specific tests before or after the scan. None shared any equipment or locations with carriers, none received multidose injectable medications or contaminated saline flushes, and none shared other exposures during their CT procedure. All patients had recently undergone a CT scan with contrast: 3 patients on June 11, and 1 each on June 25, June 29, and August 9. An epidemiologic study was conducted that defined a hepatitis C case as a person who satisfied the following 3 criteria: 1) diagnosis of acute hepatitis C according to the case definition of the Catalan Surveillance System (*13*); 2) having undergone a CT scan with contrast in the 6 months before diagnosis; and 3) the case was detected from August through November 2004. All persons tested with the same multidose contrast equipment; a reported case were screened to detect possible HCV carriers.

Transmission was demonstrated by similarity of HCV sequences isolated from acute hepatitis cases with those sequences isolated from carriers with chronic hepatitis C by using phylogenetic analysis. Briefly, glycoprotein E2 coding sequence (nt 1301–1808 encompassing hypervariable region 1) was amplified by nested PCR as previously described (*3*,*4*). This analysis was performed in 2 laboratories: Hospital Clinic and Hospital Vall d'Hebron.

Four independent carrier-case events were identified, as shown in Table 1. Three cases on the same day were

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Table 1. Characteristics of 6 acute hepatitis C infections attributable to computed tomography (CT) scan with contrast*

Characteristic	Event 1			Event 2	Event 3	Event 4
Hospital/center	A	A	A	A	B	C
Carrier no.	1	1	1	2	3	4
CT scan date/time	2004 Jun 11, 3:00 PM	2004 Jun 11, 3:00 PM	2004 Jun 11, 3:00 PM	2004 Jun 25, 9:05 AM	2004 Jun 29, 11:00 AM	2004 Aug 9, 5:44 PM
Case-patient no.	1	2	3	4	5	6
Sex	F	M	F	F	M	M
Age, y	47	58	57	55	29	61
CT scan date/time	2004 Jun 11, 3:50 PM	2004 Jun 11, 4:16 PM	2004 Jun 11, 5:17 PM	2004 Jun 25, 9:37 AM	2004 Jun 29, 11:29 AM	2004 Aug 9, 6:43 PM
Symptom onset	2004 Aug 7	2004 Aug 8	Asymptomatic	2004 Aug 4	2004 Aug 12	2004 Aug 18
Diagnosis	2004 Aug 9	2004 Aug 12	2004 Oct 15	2004 Aug 14	2004 Aug 18	2004 Aug 28
Diagnostic test	Seroconversion, anti-HCV, RNA HCV	Seroconversion, anti-HCV, RNA HCV	Seroconversion, anti-HCV, RNA HCV	Anti-HCV, RNA HCV	Seroconversion, anti-HCV, RNA HCV	Seroconversion, anti-HCV, RNA HCV
Date reported	2004 Aug 20	2004 Aug 20	NA	2004 Aug 20	2004 Nov 22	2004 Nov 5
Genotype	1b, phylogenetic identity with carrier 1	1b, phylogenetic identity with carrier 1	1b, phylogenetic identity with carrier 1	1b, phylogenetic identity with carrier 2	1b, phylogenetic identity with carrier 3	1b, phylogenetic identity with carrier 4

*The 2 hospitals and 1 diagnostic center from which case-patients were identified are designated as A, B, and C. Anti-HCV, antibody to hepatitis C virus; NA, not applicable.

related to the same carrier, 2 cases were identified immediately after the carrier, and 1 case (asymptomatic) was not consecutive (only 1 person between cases 2 and 3), resulting in a secondary attack rate of 60.0% (95% confidence interval [CI) 17.04–92.74) (Table 2) In the other 3 events, the case was scanned immediately after the carrier; global secondary attack rate was 26.08% (95% CI 11.08–48.68). The first event was probably caused by a carrier viral load greater than that of the other events. All cases involved the immediately prior scan of an HCV carrier and they clustered in the same node, on the basis of 1,000 resamplings, with a value ≈100%; some part of the contrast injection equipment had been used for all cases (Figure 1). All transmission events resulted in well-defined, highly supported monophyletic groups when samples involved in the outbreak were analyzed (>95%). Conversely, sequences from unrelated samples did not show bootstrap support (<70%) (*3*,*14*). Epidemiologically unrelated strains from the same area were included in the analysis.

In the hospital where 2 episodes of transmission occurred on different days, a retrospective study was carried out to eliminate other cases of transmission of HCV among the 1,486 patients subjected to a CT scan with contrast after a patient with known HCV infection from May 15 through August 15, 2004. This period included the exposure period of both transmissions. This analysis yielded 71 infectious HCV carriers who had a scan in which equipment was shared with noninfected patients. Of the 71 pairings of carriers-susceptible patients, 57 susceptible persons were tested for antibodies to HCV, 10 had died, and 4 could not be located. No new cases were identified, resulting in a frequency of HCV transmission of 3.5 95% CI 0.61–13.16) per 100 carriers for whom equipment was shared during the 3-month period. The consecutively scanned patient was HCV negative.

Characteristics of the equipment used for contrast injection in all settings were investigated, along with the procedure used, to identify critical points for virus transmission. The equipment (Figure 2) included a contrast injector with automatic load from a 500-mL bottle that was shared by ≥4 persons. The contrast solution arrived in a prefilled bottle (manufactured by different pharmaceutical companies) that is loaded through a connection tube into the injector. We investigated this procedure and did not detect any risk of

Table 2. Transmission of hepatitis C during computed tomography scan with contrast*

Characteristic	2004			
	Jun 11	Jun 25	Jun 29	Aug 9
No. patients scanned after carrier, sharing same equipment	5	11	5	2
No. cases of transmission	3	1	1	1
Time between carrier scan and case scan	2 h 17 min	32 min	29 min	59 min
Hospital	A	A	B	C
Secondary attack rate, % (95% CI)	60 (17.04–92.74)	9.09 (0.47–42.88)	20 (1.05–70.12)	50 (2.66–97.33)
Syringe replacement	Every 8 h	Every 8 h	≤8 h	≥24 h
Disconnection procedure with risk of transmission	Present	Present	Present	Present

*CI, confidence interval.

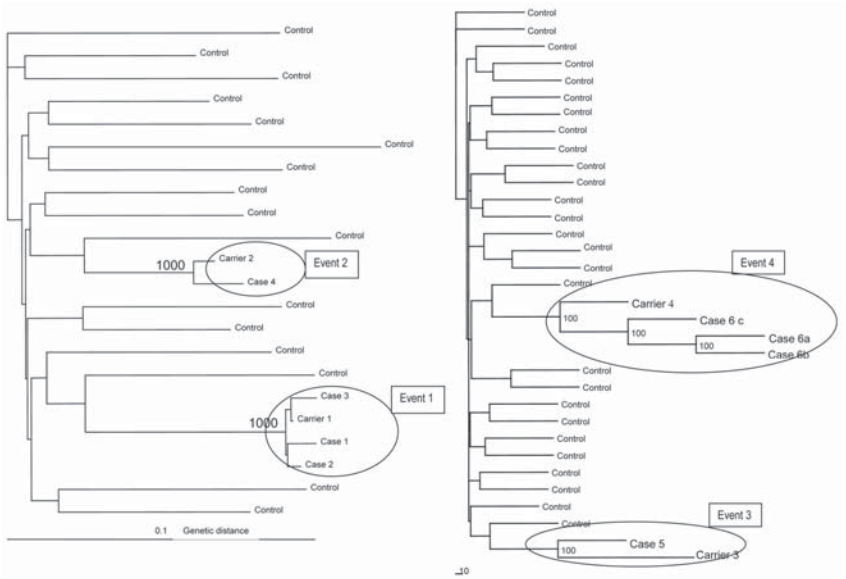

Figure 1. Phylogenetic tree of the partial glycoprotein E2 sequences of hepatitis C virus from patients investigated in 2 public hospitals and 1 private diagnostic center and control samples retrieved from Hospital Clinic and Hospital Vall d'Hebron in Barcelona, Spain. GenBank accession nos.: DQ682391, DQ682392, DQ682393, DQ682394, DQ682376, DQ682377, EU380670, EU380671, EU380672, EU380673, EU380674, EU380675. Branch lengths are drawn to scale. Only bootstrap values >70% are shown.

blood contamination. Replacement of the injector varied among hospitals, and use time ranged from 8 hours to several days. All equipment was connected to the patient by the intravenous route through an extension tube fitted with a nonreturn valve. This extension tube was the only part of the equipment that was changed for each patient. One maneuver involving risk of blood contamination of the extension tube was identified in all hospitals. Contamination could have occurred through the hands of health personnel manipulating the extension tube by disconnecting the tube from the patient first and then from the equipment without changing gloves between these manipulations. Other factors that could have contributed to transmission were lack of written instructions to guarantee proper practice; training of new technicians by experienced technicians, which may perpetuate errors; and work overload, with a maximum time of ≤9 min available for each CT scan (from the time the patient enters until he or she leaves), which could lead to a lower level of standard precautions.

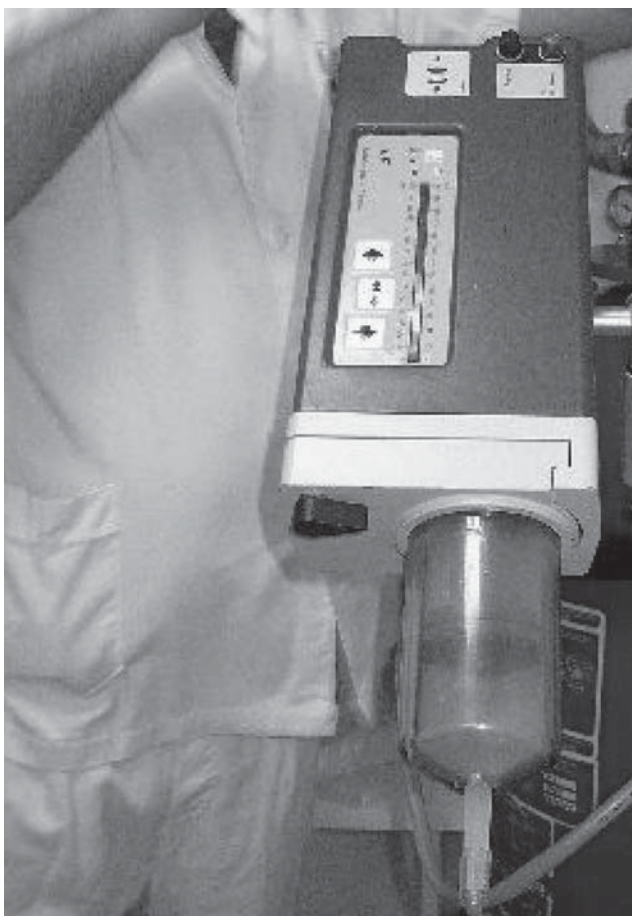

Figure 2. Equipment for contrast administration in computed tomography scan.

Two sets of control measures were used. The first set, which emphasized appropriate use of health products and stressed the importance of following manufacturer's instructions (a contrast injection syringe for each patient), was obtained from the Agencia Española del Medicamento. The second set included a written protocol for performing a CT scan with contrast and procedures to avoid transmission of infectious diseases. A follow up of infected patients was ensured, as well as preventive treatment. This will be reported by the responsible team in the future.

Conclusions

Our study had several limitations that should be taken into account. First, our study was a case series and we only described cases and their potential sources of infection. Second, although we carried out active case finding, we must assume that other related cases were not detected. Detection of this problem in 3 hospitals in the same city

within a short period suggests that CT scan with contrast could be responsible for an unknown number of preventable HCV infections in industrialized countries if infection control procedures are not strictly applied.

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Chikungunya Fever, Mauritius, 2006

To the Editor: When an outbreak of chikungunya fever swept across several Indian Ocean islands in 2005 and 2006, the international public health community's attention was drawn to chikungunya virus (genus *Alphavirus*, family *Togaviridae*). Among those areas affected was the Republic of Mauritius, located ≈900 km east of Madagascar, which has an estimated population of 1,250,000 and comprises the main island of Mauritius (area ≈1,865 km^2) and several outlying islands including Rodrigues (≈180 km^2). Mauritius Island had an initial outbreak of ≈3,500 suspected cases of chikungunya fever from April through June 2005 (*1*). With the onset of the drier season of winter, transmission of the virus subsided, but increased again during 2006. An outbreak began on Rodrigues Island in February 2006. The intensity of chikungunya fever outbreaks on Mauritius and Rodrigues Islands led us to explore the extent to which these outbreaks might have contributed to overall death rates.

Chikungunya virus is transmitted to humans primarily by the bite of infected *Aedes* spp. mosquitoes. *Aedes aegypti* was effectively eliminated from the island of Mauritius during a malaria control campaign from 1949 through 1951 (*2*). However, *A. albopictus* is widely distributed on the island in rural and urban habitats (*3*); thus, *A. albopictus* may be the most likely vector for chikungunya virus. Common peridomestic breeding places in Mauritius include small pots and vases (used to decorate the outside of homes) and discarded rubber tires (*3*).

Chikungunya fever was first recognized in 1952 after an outbreak on the Makonde Plateau of Tanganyika Territory (*4*), currently part of Tanzania. The word *chikungunya* is from a local dialect that translates to "that which bends up" in reference to the stooped posture often seen in patients with severe arthralgia (*5*). Human infections with chikungunya virus are associated with sudden onset of symptoms including headache, fever, rash, and muscle and joint pain (*5*). Before the outbreak in the Indian Ocean islands, deaths had not been associated with infections. However, Réunion Island reported >200 deaths during the 2006 chikungunya epidemic (January–April 2006) (*6*), and India conservatively estimated 1,194 deaths since the virus reemerged in December 2005. (*7*). To determine whether a similar situation occurred in Mauritius, we compared expected number of deaths with observed number of deaths and estimated number of chikungunya cases from January through December 2006.

Crude death rates (CDRs) and number of deaths were obtained from the Annual Digest of Statistics 2005 and 2006 (*8*). We calculated expected number of deaths for each month in 2006 by multiplying mean CDR (per 1,000 midyear population) of each month for the previous 10 years (1996–2005) by midyear population for 2006. Estimated number of cases of chikungunya fever in 2006 for the Island of Mauritius was obtained from the Chikungunya Unit of the Ministry of Health and Quality of Life and includes both suspected and confirmed cases. A case was considered suspected if the patient had ≥2 of the following 3 symptoms: fever, rash, and joint pain. Cases were confirmed by cell culture.

CDRs for the Republic of Mauritius ranged from 6.7 to 7.0 during 1996–2005 (mean 6.8) but increased to 7.3 in 2006, which represented a 4.3% (95% confidence interval [CI] 2.2%–8.5%) increase from 2005 (CDR 7.0). According to the 2006 midyear Population and Vital Statistics Report for the Republic of Mauritius (*8*), the number of deaths from January through June 2006 increased by 16.7% (95% CI 11.0%–23.5%) over the number of deaths in 2005, and 19.8% (95% CI 13.5%–26.9%) more deaths occurred among elderly persons (age ≥60 years). These numbers include all potential causes of deaths. However, increased CDRs occurred only during the months of the chikungunya fever epidemic on Rodrigues and Mauritius Islands.

CDRs on the Island of Mauritius in March, April, and May 2006 were significantly greater than expected ($p<0.01$ for all 3 months); this corresponds to 743 excess deaths for these months (Figure). The chikungunya fever epidemic in Mauritius began ≈1 month before the first month of reported excess deaths; 4,871 cases of chikungunya fever cases were reported in February, 5,084 in March, and 2,305 in April. Rodrigues Island showed a similar trend, with an increase in the number of observed deaths in April, May, and June ($p<0.01$ for May).

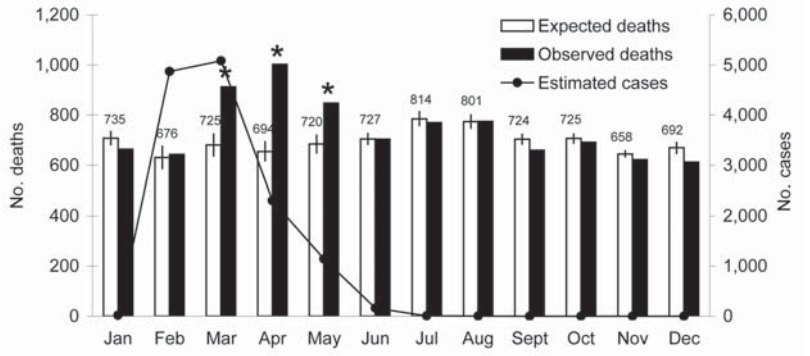

Figure. Expected and observed numbers of deaths and estimated number of chikungunya cases on Mauritius Island. Asterisks indicate a statistically significant ($p<0.01$) difference between expected and observed number of deaths for the specified months. Vertical lines show 95% confidence intervals; upper confidence limits are shown numerically.

The first confirmed case of chikungunya fever on Rodrigues Island was reported in February, followed by 56 cases in March, 393 in April, and >80 in May (*9*).

The increase in CDRs reported for the Republic of Mauritius during the chikungunya epidemic is similar to the findings reported for the neighboring island of Réunion (*10*). Excess deaths in Réunion and the Republic of Mauritius coincided with the epidemic curve of the chikungunya fever outbreak, which suggested an association between these 2 factors. No other events that may have negatively affected the health of persons living in the Republic of Mauritius in 2006 were reported. However, because information on cause of death is unavailable, studies are needed to confirm that the chikungunya fever outbreak contributed to increased CDRs in 2006.

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Increasing Resistance in Commensal *Escherichia coli*, Bolivia and Peru

To the Editor: The global increase of antimicrobial-drug resistance, including resistance to the new and most potent antimicrobial agents, is a major public health concern. In low-resource countries, where bacterial infections are still among the major causes of death, especially for children, it is of particular concern (*1*).

ANTRES (Towards Controlling Antimicrobial Use and Resistance in Low-income Countries—An Intervention Study in Latin America) is a research project on antimicrobial-drug use and resistance in low-resource countries of Latin America (see www.unifi.it/infdis/antres/default.htm). In 2002, the baseline ANTRES study showed a high rate of fecal carriage of *Escherichia coli* with acquired resistance to several antimicrobial drugs, especially older drugs (e.g., ampicillin, trimethoprim-sulfamethoxazole, tetracycline, streptomycin, and chloramphenicol), in preschool children from 4 urban settings in Bolivia and Peru (*2*). We report the results of a second cross-sectional study, conducted in 2005, that evaluated the evolution of antimicrobial-drug resistance in the studied areas.

We studied healthy children 6–72 months of age from each of 4 urban areas: 2 in Bolivia (Camiri, Santa Cruz Department; Villa Montes, Tarija Department) and 2 in Peru (Yurimaguas, Loreto Department; Moyobamba, San Martin Department). The study design, sampling and inclusion criteria, methods, and ethical issues were the same as those of the baseline study (*2*). The study was carried out over 4 months (September–December 2005), the same seasonal period as in the previous study. No significant differences in sex ratios were found among children enrolled from the different areas,

whereas minor differences were found for age. No statistical differences were found between the 2002 baseline study and the 2005 study results in terms of numbers of children (3,193 vs. 3,174) and sex ratios (0.94 vs. 0.95) (Table). Statistical analyses were performed by using Stata 9.0 (Stata Corp., College Station, TX, USA). Logistic regression models were used to compare the antimicrobial-drug resistance rates in 2002 and 2005, considering the combined influences of age, sex, city, and country.

Data from the 2005 survey confirmed high resistance rates for ampicillin, trimethoprim-sulfamethoxazole, tetracycline, streptomycin, and chloramphenicol. The differences in resistance rates observed between 2002 and 2005 for these drugs, although sometimes statistically significant, are probably of limited epidemiologic relevance due to the high rates of antimicrobial-drug resistance found in the *E. coli* population in both surveys. The most relevant finding of the 2005 survey was the remarkable increase since 2002 in the resistance rates to fluoroquinolones and expanded-spectrum cephalosporins (Table). Molecular analysis showed that the dramatic increase in rates of resistance to expanded spectrum cephalosporins was mostly the result of dissemination of CTX-M-type extended-spectrum β-lactamase determinants (*3*). Concerning the association between sex and resistance rates, the higher resistance rates observed for some agents and in some settings for boys in the baseline study were not confirmed, except in 1 case (kanamycin in Camiri, p = 0.04) (*2*). Analysis by age (not performed for amikacin due to low numbers of resistant isolates) confirmed the occurrence of higher resistance rates for the youngest age group and an overall decreasing trend by age for all agents, except ciprofloxacin and gentamicin. For these 2 agents, resistance rates increased, although not significantly (p = 0.95 and p = 0.55, respectively) (*2*). Although we did not specifically address factors potentially involved in this phenomenon, we will address them in future investigations.

Increasing resistance to fluoroquinolones and expanded-spectrum cephalosporins among *E. coli* clinical isolates has been observed in several parts of the world and complicates the management of infections (*4*,*5*). Recently, intestinal colonization with fluoroquinolone-resistant or extended-spectrum β-lactamase–producing *E. coli* of nonhospitalized persons has been described as an emerging phenomenon (*6*–*9*). Although the exact clinical implications of this phenomenon are not clearly established, colonization by these resistant strains is a public health threat at the community and hospital levels (*8*,*9*). The reasons for the increased prevalence of fecal carriage of these resistant *E. coli* strains by children from the studied areas are not clear. Data collected about household use of antimicrobial drugs excluded previous use of fluoroquinolones and expanded-spectrum cephalosporins (C. Kristiansson et al., unpub. data). The increased prevalence of resistant *E. coli* strains in preschool children most likely reflects increased exposure within a contaminated household setting, in the food chain, or both (*6*,*8*,*10*).

Our findings support the need to continue monitoring the evolution of resistance in commensal *E. coli*, to evaluate the effects of these important reservoirs of resistance genes distributed in the community, to investigate the epidemiologic relationship with clinical isolates, and to define the role of the food supply. Investigation into whether carriage of resistant strains in adults correlates with data on antimicrobial-drug use in hospitals and in the community would also be of interest.

Table. Antimicrobial drug–resistance rates of *Escherichia coli* as part of commensal flora in children, Bolivia and Peru, 2002 and 2005*

Drug†	2002	2005	p value‡
AMP	95	96	<0.05
CRO	0.1	1.7	<0.001
TET	93	93	NS
SXT	94	94	NS
CHL	70	69	NS
STR	82	92	<0.001
KAN	28	29	<0.05
GEN	21	27	<0.001
AMK	0.4	0.1	NA
NAL	35	57	<0.001
CIP	18	33	<0.001

*Expanded Table available online at www.cdc.gov/EID/content/14/2/338-T.htm. Prevalence expressed as percentages. In 2002, n = 3,174, mean age 34.8 mo; in 2005, n = 3,193, mean age 33.7 mo (mean age p<0.05).
†AMP, ampicillin; CRO, ceftriaxone; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; STR, streptomycin; KAN, kanamycin; GEN, gentamicin; AMK, amikacin; NAL, nalidixic acid; CIP, ciprofloxacin.
‡Wald test applied to establish the statistical significance of parameters obtained from logistic regression analysis; NS, not significant; NA, not applicable (due to lack of variability of data).

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Plasmid-mediated Quinolone Resistance in *Salmonella enterica*, United Kingdom

To the Editor: Fluoroquinolones are broad-spectrum antimicrobial drugs used to treat many clinical infections. Salmonellosis is treated with fluoroquinolones only in elderly or immunocompromised patients, but these drugs are also used for treating patients with enteric fever, invasive disease, or long-term salmonellae carriage. High-level fluoroquinolone resistance is uncommon, but reduced susceptibility is increasing.

Since 1998, plasmid-mediated quinolone resistance encoded by *qnr* genes A, B, and S that confer low-level resistance to nalidixic acid and reduced susceptibility to ciprofloxacin has been identified in several enterobacterial species, including *Salmonella*. Their clinical importance is in facilitating resistance to potentially lethal levels of quinolone. Additionally, *qnr* genes are often associated with strains that produce extended-spectrum β-lactamases.

We recently reported identification of *qnr* genes in *Salmonella* in the United Kingdom (*1*). Most isolates were associated with the Far East. Two isolates of *S.* Virchow were part of an outbreak associated with imported cooked chicken from Thailand. During October 2006–April 2007, we monitored *qnr* genes in nontyphoidal salmonellae isolated in the United Kingdom that expressed reduced susceptibility to ciprofloxacin (MIC 0.125–1.0 μg/mL) with concomitant susceptibility to nalidixic acid (MIC <16 μg/mL). This resistance phenotype is a useful marker for the *qnr* gene as the sole quinolone resistance determinant (*1*).

Recent studies showed that isolates of *Salmonella* spp. and *Escherichia coli* with decreased susceptibility to ciprofloxacin (MICs >0.06 μg/mL and 0.5 μg/mL, respectively), but with susceptibility or intermediate resistance to nalidixic acid (MIC 8–16 μg/mL and 4–8 μg/mL, respectively), all had *qnrA* or *qnrS* genes but lacked mutations in the topoisomerase genes (*2*,*3*). Strains with ciprofloxacin MICs >1 μg/mL were also included to monitor involvement of *qnr* genes in development of high-level ciprofloxacin resistance. Breakpoint concentrations used are based on long-term studies within the Health Protection Agency Laboratory of Enteric Pathogens. Ciprofloxacin Etest (AB Biodisk, Solna, Sweden) results were interpreted according to manufacturer's procedures. A total of 45 *Salmonella* spp. strains were tested. Screening for *qnr* genes by multiplex PCR identified 37 isolates with *qnrS* and 2 carrying *qnrB* variants (Table) (*4*). However, the *qnrB* primer pair in this multiplex did not fully match all *qnrB* gene variants. PCR and sequencing using primers FQ1 and FQ2 (*5*) and *qnrS-F* and *qnrS-R* (*1*), were used to identify specific *qnrB* and *qnrS* gene variants.

The *qnrS1*-positive salmonellae belong to serotypes Typhimurium

Table. Isolates of *Salmonella enterica* with plasmid-mediated *qnr* genes, United Kingdom, October 2006–April 2007

Salmonella serotype	Phage type*	No. isolates	VNTR profile†	Ciprofloxacin MIC (μg/mL)‡	Additional resistance to antimicrobial drugs§	*qnr* identified
Corvallis	–	1	–	0.25	S, Su, T	*qnrS1*
Corvallis	–	2	–	0.38	S, Su, T	*qnrS1*
Corvallis	–	1	–	1.0	S, Su, T ,Cf	*qnrS1*
Corvallis	–	1	–	0.25	None	*qnrS1*
Corvallis	–	1	–	0.38	None	*qnrS1*
Schwarzengrund	–	1	–	0.25	T	*qnrB5*
Typhimurium	DT120	4	1–6-0–0-3	0.38	S, Su, T	*qnrS1*
Typhimurium	DT120	3	1–6-0–0-3	0.50	S, Su, T	*qnrS1*
Typhimurium	DT120	3	1–4-0–0-3	0.38	S, Su, T	*qnrS1*
Typhimurium	DT120	1	1–4-0–0-3	0.50	S, Su, T	*qnrS1*
Typhimurium	DT120	1	1–4-0–0-3	0.38	None	*qnrS1*
Typhimurium	DT120	1	1–5-0–0-3	0.38	S, Su, T	*qnrS1*
Typhimurium	DT193	1	1–6-0–0-3	0.50	S, Su, T	*qnrS1*
Typhimurium	DT193	1	1–4-0–0-3	0.38	C, S, Su, Sp, T, Tm	*qnrS1*
Typhimurium	DT193	1	1–4-0–0-3	0.38	S, Su, T	*qnrS1*
Typhimurium	DT193	2	1–5-0–0-3	0.38	S, Su, T	*qnrS1*
Typhimurium	DT193	1	1–4-0–0-3	0.50	A, Su	*qnrS1*
Typhimurium	49b	1	1–4-19–1-3	0.25	A, G, Ne, K, S, Su, Sp, T, Tm, Ak, Cx, Cr, Cf, Cn, Ct	*qnrB2*
Typhimurium	NC	1	1–4-0–0-3	0.25	S, Su, T	*qnrS1*
Typhimurium	UT	1	3–8-19–1-2	>32	A, C, G, S, Su, Sp, T, Tm, Fu, Nx	*qnrS1*
Virchow	43	5	–	1.0	A, Fu, Nx	*qnrS1*
Virchow	43	2	–	1.5	A, Fu, Nx	*qnrS1*
Virchow	25a	1	–	0.75	Tm	*qnrS1*
Virchow	11	1	–	1.0	A, Fu, Nx	*qnrS1*
Virchow	NC	1	–	1.5	A, C, G, Ne, K, S, Su, Sp, T, Tm, Fu, Nx, Cx, Cr, Cf, Cn, Ct	*qnrS1*

*DT, definitive type; NC, does not conform to a recognized pattern; UT, untypeable.
†VNTR, variable number tandem repeat. Loci of the VNTR profiles are presented in the following order: STTR9-STTR5-STTR6-STTR10pl-STTR3. The number 0 in the VNTR profile represents cases with no amplification of PCR product.
‡Determined by Etest.
§Antimicrobial drugs (breakpoint final concentrations): S, streptomycin (16 mg/L); Su, sulfonamide (64 mg/L); T, tetracycline (8 mg/L); Cf, cefuroxime (16 mg/L); C, chloramphenicol (8 mg/L); Sp, spectinomycin (64 mg/L); Tm, trimethoprim (2 mg/L); A, ampicillin (8 mg/L]; G, gentamicin (4 mg/L); Ne, neomycin (8 mg/L) K, kanamycin (8 mg/L); Ak, amikacin (4 mg/L); Cx, cefalexin (16 mg/L); Cr, cefradine (16 mg/L); Cn, ceftriaxone (1 mg/L); Ct, cefotaxime (1 mg/L); Fu, furazolidone (8 mg/L); Nx, nalidixic acid (16 mg/L).

(21 isolates), Virchow (10), and Corvallis (6). Most *S.* Typhimurium isolates were either definitive phage type 120 or 193, and most *S.* Virchow isolates were phage type 43 (Table). Thirteen *qnrS1*-positive isolates were from patients who reported recent travel to Egypt, India, Malaysia, Morocco, Thailand, or an undisclosed destination.

Twelve isolates from patients who had not traveled abroad were assumed to be from UK-acquired infections. *S.* Virchow isolates had been associated with cooked chicken from Thailand (*1*), and *qnrS1* has recently been described in *S.* Corvallis strains from humans in Denmark or isolated in Thailand from humans, chicken, pork, and beef (*3*). Comparison of pulsed-field gel electrophoresis patterns and resistance phenotypes of *qnrS1*-positive *S.* Corvallis strains identified common types, suggesting that some UK patients may have acquired *S.* Corvallis from chicken from Thailand.

Thirteen isolates showed resistance to ceftriaxone, cefotaxime, or ampicillin. Plasmids with *qnr* genes have been found to co-transfer TEM, SHV, and CTX-M genes (*1*,*5*,*6*). Co-transmission of fluoroquinolone and β-lactamase resistance is clinically important because co-selection of resistance by use of either drug may occur.

Twenty-one *qnrS1*-positive *S.* Typhimurium were subtyped by variable number tandem repeat (VNTR) analysis to determine whether the increase was caused by spread of ≥1 distinct strains (*7*). Twenty isolates produced 1 of 3 related profiles (loci of VNTR profiles are ordered STTR9-STTR5-STTR6-STTR10pl-STTR3): 1–4-0–0-3, 9 isolates; 1–5-0–0-3, 3 isolates; or 1–6-0–0-3, 8 isolates. Alleles 4 and 5, and 5 and 6 at locus STTR5 only differed by an extra 6-bp repeat, which suggests a clonal relationship between the *qnrS1*-positive *S.* Typhimurium in this study (Table) (*8*). *S.* Typhimurium isolates with the 1–6-0–0-3 profile have been isolated from tourists returning from Asia (*7*), which suggests that the UK *qnrS1*-positive *S.* Typhimurium isolates have originated in the Far East.

These findings show increased occurrence of *qnr* genes, particularly

qnrS1, in nontyphoidal salmonellae in the United Kingdom. These data are in contrast to those of recent studies in the United States and France, which show low incidences of *qnrS* genes in larger strain collections (*9*,*10*). The *qnr* phenotype is in contrast to resistance mediated by mutations in the topoisomerase genes whereby 1 mutation confers low-level resistance to fluoroquinolones and full resistance to nalidixic acid. Our previous study demonstrated that *qnrS1* was sufficient to cause decreased susceptibility to ciprofloxacin in the absence of mutations in *gyrA* (*1*). In this study, a *qnr* gene was sufficient to increase the ciprofloxacin MIC to 0.38–0.75 μg/mL. In addition, a *qnr* gene contributed to high-level ciprofloxacin resistance in 10 isolates, thereby potentially jeopardizing first-line treatment of vulnerable patient groups with ciprofloxacin.

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Saksenaea vasiformis Infection, French Guiana

To the Editor: The Zygomycetes are a class of filamentous fungi that are ubiquitous in the environment. Most of the species known to cause human or animal infections belong to a few genera within the order Mucorales. *Saksenaea vasiformis*, isolated from soil in India and described by Saksena in 1953, was reported to cause human infection for the first time by Ajello et al. (*1*). We report a case of a cutaneous lesion caused by *S. vasiformis* in French Guiana.

A nonimmunocompromised 47-year-old woman with a long history of non–type 1 diabetes mellitus, who had lived in French Guiana for many years, was admitted to Cayenne Hospital on November 18, 2005, with a cutaneous lesion of the abdominal wall and a fever that had lasted for 5 days before she was hospitalized. A skin biopsy specimen was obtained, and the first surgical debridement was performed on day 4 of hospitalization. A diagnosis of zygomycosis was made after direct examination and histopathologic examination of the tissue samples. Treatment was initiated on day 8, beginning with liposomal amphotericin B and itraconazole for 10 days, followed by liposomal amphotericin B alone for 12 days. Persistence of necrotic tissues at the infection site required additional surgical debridement on day 10. Histopathologic examination of the resected tissues showed damaged hyphae of zygomycetes. Resolution of clinical signs was excellent. Additional biopsy specimens taken by the end of treatment on day 21 were negative for fungi by direct examination and culture. Finally, a cicatrix was formed.

Histologic examination of the initial excised tissues showed a localized periumbilical cutaneous lesion of

14 cm × 13 cm. The skin was covered by a 1-mm layer of necrosis. The necrosis extended into all the abdominal adipose tissue at the rectus abdominis muscle and linea alba. Microscopy examination showed extensive superficial mycotic proliferation, with wide and irregular ribbonlike nonseptate hyphae and right-angle branching. These hyphae extended toward the hypodermic fat tissues and were associated with a break in the cell membrane of adipocytes and with crystals inside the adipocytes. These lesions were associated with massive nonsuppurative vascular thrombosis.

Culture of tissues samples on Sabouraud-chloramphenicol-gentamicin agar after 4 days at 30°C and 37°C grew a white aerial mold, which covered the entire surface of the agar. Examination by microscopy showed nonseptate sterile hyphae typical of a zygomycete. The fungal isolate was sent to the National Reference Center for Mycology and Antifungals at the Institut Pasteur, Paris. Subcultures on different media including malt extract agar and potato dextrose agar grew sterile mycelia. The isolates were then cultured in nutritionally deficient medium consisting of sterile distilled water supplemented with 0.05% filter-sterilized yeast extract (Difco, Becton, Dickinson and Company, Sparks, MD, USA) solution for 7 days at 37°C (*2*). Typical flask-shaped sporangia enabled identification of *S. vasiformis* (Figure). Sporulation also occurred on Czapek agar after 7 days' incubation at 37°C.

Molecular identification based on PCR amplification and sequencing of rDNA internal transcribed spacer (ITS) regions was also performed. Briefly, mycelia were grown in liquid Roswell Park Memorial Institute medium, and DNA was extracted as previously described (*3*). Ribosomal DNA, including the complete ITS1–5.8S-ITS2 region, was amplified with the fungal universal primer pairs V9D/LS266 (*4*) and ITS1/ITS4 (*5*), and both strands of PCR products were sequenced. The sequence has been deposited in GenBank (accession no. EU182902). Sequence alignment with the only *S. vasiformis* ITS sequence available in the GenBank database showed 82% similarity over 530 bp. This low degree of sequence homology is probably reflective of the need for further phylogenetic study of this genus.

Sporulation on Czapek agar enabled preparation of a sporangiospore suspension used for antifungal susceptibility testing. Sporangiospore suspension was counted microscopically and adjusted to the required density. MICs, determined by the EUCAST reference microdilution method (*6*), after 48 h of incubation were >8, 2, >8, 0.5, and >8 μg/mL for amphotericin B, itraconazole, voriconazole, posaconazole, and caspofungin, respectively. The MIC of 0.5 μg/mL for posaconazole suggests the potential clinical utility of this agent.

S. vasiformis has been isolated from soil samples in different parts of the world (*7*). This fungus has been rarely responsible for human infections. A recent review (*8*), which did not include infrequently cited articles (*9*,*10*), found only 30 human cases. This scarcity may occur because the diagnosis is often based on histologic features and *S. vasiformis* does not sporulate in routine mycology media.

Due to zygomycetes' lack of susceptibility to most of the antifungal agents, identification of a zygomycete as the etiologic agent of an infection is essential for rapid and accurate management of the disease. Rare Zygomycetes species such as *S. vasiformis* or *Apophysomyces elegans* should be suspected when a nonsporulating zygomycete is isolated from an infected lesion. When this acute infection is suspected after examination of tissue by microscopy, the fungi should be cultured in specific culture media to induce sporulation or they should be identified by molecular tools.

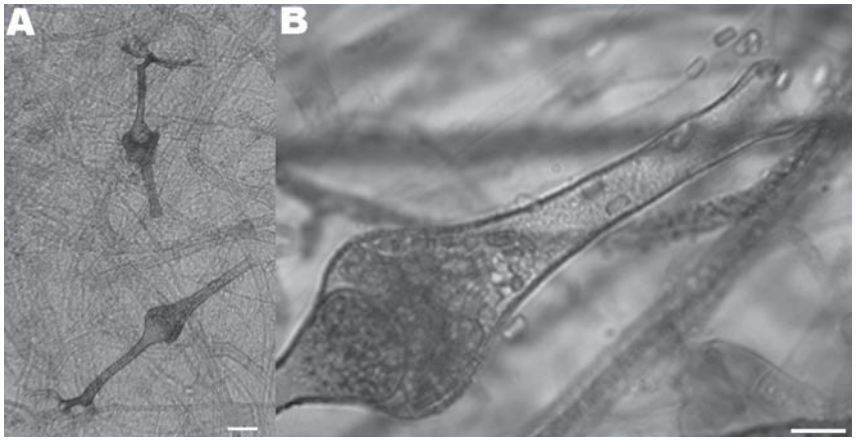

Figure. Microscopic characteristics of the isolate of *Saksenaea vasiformis* cultured on Czapek agar. A) Typical flask-shaped sporangia (scale bar = 25 μm) containing B) smooth-walled, rectangular sporangiospores (scale bar = 10 μm).

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Q Fever in Young Children, Ghana

To the Editor: Recently, experts identified Q fever, caused by the small, gram-negative bacterium *Coxiella burnetii*, as an important underdiagnosed childhood disease (*1*). Studies on Q fever in children <5 years of age are scarce, especially with respect to sub-Saharan Africa. The only available study from Niger reports a seroprevalence of 9.6% (*2*). Throughout Africa, prevalence of Q fever in adults shows considerable variability and is highest in countries with prominent stockbreeding (*3*).

Clinical manifestations of Q fever in children are similar to those of malaria (*1*,*4*). In malaria-endemic areas, most fevers are attributed to *Plasmodium falciparum* infection and presumptively treated with expensive combination therapies (*5*). In this context, other neglected fever-causing pathogens need to be given appropriate consideration.

We studied the prevalence of Q fever antibodies in 219 randomly selected children living in 9 rural villages of the Ashanti region, Ghana. Plasma was obtained by venous puncture from 2-year-old children after they had participated in a malaria control study and had been clinically monitored for 21 months. Clinical, parasitologic, socioeconomic, and Global Positioning System information was recorded as described elsewhere (*6*,*7*). In addition, 158 healthy adult volunteers from the same area were included. Plasma was stored at –20°C until microimmunofluorescence assays (IFA) (*Coxiella burnetii* I+II, Vircell SL Microbiologists, Granada, Spain) were performed according to manufacturer's instructions. To identify all children with Q fever titers, we regarded the following as positive fluorescence reactions to plasma dilutions: ≥1:64 for phase II immunoglobulin (Ig) G and ≥1:24 for phase II IgM with sensitivity (specificity) of 97.2% (100%) and 100% (56.3%), respectively. IgM testing was only performed on IgG-positive children. Positive and negative controls were run on each IFA slide. Relative risks (RR) for characteristics of children were calculated by χ^2 test; $p<0.05$ was considered significant. Informed consent was obtained from all participants or their parents. The study protocol was approved by the committee on human research and publication, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Positive *C. burnetii* phase II IgG responses were observed in 37 (16.9%) of 219 children and 14 (8.9%) of 158 adults (Figure, panels A and B). In comparison to adults, more children had IgG titers ≥64 (Figure, panels C and D). On the day of the serosurvey 71 (32.4%) of 219 children had fever (measured body temperature >38°C or reported fever within the previous 48 hours). Test outcome did not appear to be influenced by *P. falciparum* infection, since 4 of 37 IgG-positive children (23 of 182 IgG-negative children) had clinical malaria, 11/37 (62/182) had asymptomatic parasitemia, and 6/37 (38/182) had fever without parasitemia, and there were no significant differences between groups. The frequency of prior malaria episodes also did not influence antibody response. Three aparasitemic children had positive phase II IgM titers (24, 96, and 1,536; phase II IgG 64, 64, and 4,096, respectively). The child with the high IgM and IgG titers was clinically ill with nonsevere *C. burnetii* pneumonia. This child was among 10 (27%) of 37 phase II IgG-positive children with detectable anti–*C. burnetii* phase I antibodies. Of all sociodemographic characteristics under consideration, only maternal illiteracy was associated with positive phase II IgG testing (RR 2.1, 95% confidence interval 1.0–4.2, $p<0.05$).

A considerable proportion of Ghanaian children had anti–*C. burnetii* antibodies, which indicates that Q fever might be a common event in

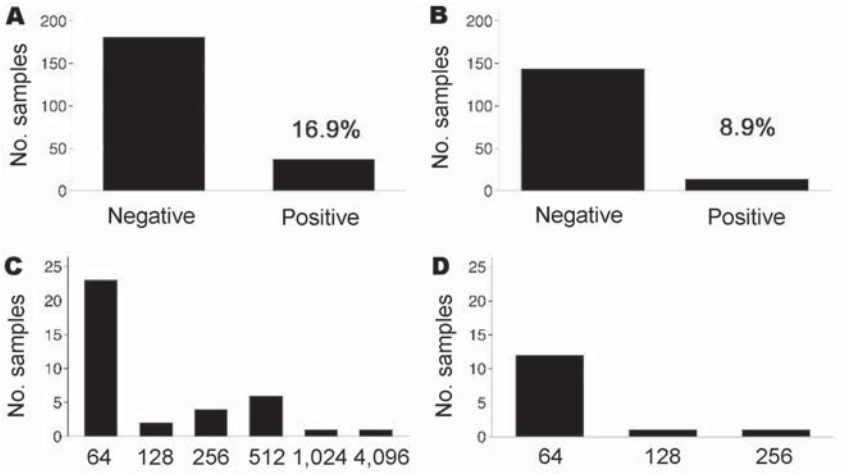

Figure. Seroprevalence of immunoglobulin (Ig) G antibodies against *Coxiella burnetii* phase II tested by microimmunofluorescence assays (IFA). A) Results of serologic tests of children, a cutoff titer of ≥64 for *C. burnetii* phase II IgG was applied; B) results of serologic tests of healthy adults (cutoff ≥64); C) distribution of *C. burnetii* phase II IgG titers in all positive children; D) distribution of IgG titers in all positive healthy adults.

this age group. Antibodies were more frequently detected in children than in adults. In adults, Q fever IgG antibodies reach a maximum 4–8 weeks after onset of symptoms and gradually decrease over months to finally fall below the detection limit (*8*).

A long period since infection is less likely in young children, which could result in higher seropositivity. Children, especially those of illiterate mothers, could also be more frequently exposed to the pathogen. Consumption of unpasteurized dairy products can result in infection or seroconversion without clinical disease (*9*). However, because consumption of raw milk in the Ashanti region is regarded as being uncommon by local health authorities, we consider dairy products an unlikely source of the disease. Although participants were intensively exposed to *P. falciparum,* which causes polyclonal B-cell stimulation, malaria episodes and parasitemia with and without symptoms at time of the serosurvey did not influence testing (*10*). This finding is important because commercially available test kits have only been evaluated in Europeans not exposed to parasites. We cannot completely rule out the possibility that other infectious agents, which are either only prevalent or more prevalent in African populations, could have resulted in false-positive results. Nevertheless, the test method we used and existing data on cross-reactions weaken this hypothesis (*8*).

We conclude that children in rural sub-Saharan Africa become exposed to *C. burnetii* early in life and that Q fever, which is clinically indistinguishable from malaria, may develop in an unknown proportion of them. The incidence of Q fever in relation to malaria, the route of infection, and appropriate serologic cutoffs for sub-Saharan Africa must be defined further. Currently, a prospective diagnostic study is investigating neglected infections, including human Q fever, as a cause of illness in Ghanaian children.

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Early Diagnosis of Disseminated *Mycobacterium genavense* Infection

To the Editor: Nontuberculous mycobacteria are environmental organisms that cause life-threatening diseases, particularly in immunocompromised hosts. They are increasingly recognized for causing problems in the management of solid-organ transplant recipients, due to improved diagnostic methods as well as increasing numbers and life expectancy of these patients (*1*). The slow-growing *Mycobacterium genavense* is a ubiquitous nontuberculous mycobacterium; it is reportedly isolated from tap water, pets, and the gastrointestinal tract of healthy humans (*1*,*2*). It was first recognized as a human pathogen in a patient with AIDS but has not yet been found in heart transplant recipients (*3*). We report early diagnosis of disseminated *M. genavense* infection in a heart transplant recipient.

A 37-year-old man was hospitalized in September 2001 for abdominal pain, sweats, and weight loss; he had received a heart transplant 3 years earlier. Immunosuppressive treatment, which began immediately after transplantation, consisted of tacrolimus (5 mg) and mycophenolate mofetil (2 g) daily; concurrent steroid therapy was tapered off over the next 6 months. A computed tomographic (CT) scan showed numerous large lymph nodes in his abdomen (Figure). Endoscopic examinations showed diffuse inflammation of the mucosa of the duodenum, ileum, and colon. Multiple biopsy samples were submitted for histologic analysis, culture, and molecular biological analysis. Immediate 16S rRNA gene amplification that used universal primers (*4*) and sequencing of samples taken directly from the biopsy material led to the identification of *M. genavense*. A 475-bp fragment was sequenced, and 99% homology with the gene of type strain ATCC 51234 (GenBank accession no. X60070) was found. PCR results were positive for 2 of the 4 samples tested. The molecular identification was compatible with the subsequent histologic finding of profound macrophage infiltration without granuloma and the presence of Ziehl-Neelsen–positive bacilli. Five weeks later, the molecular diagnosis was confirmed by blood cultures and cultures of the intestinal mucosa samples (Inno-LiPA Mycobacteria test, version 2, Innogenetics, Courtaboeuf, France). The direct molecular diagnosis of *M. genavense* enabled immediate treatment of the patient with the combination of moxifloxacin, ethambutol, clarithromycin, and amikacin; mycophenolate mofetil was discontinued. Clofazimine was added to the treatment regimen 3 months later, when a control CT scan showed that some of the enlarged mesenteric lymph nodes had increased further. After 5 months, the clinical signs resolved, and after 9 months, the lymph nodes were substantially smaller. CT scan results were within normal limits after 12 months of treatment; only ethambutol and clarithromycin were continued for an additional 6 months. There was no sign of *M. genavense* infection relapse 3 years after the diagnosis had been made.

Nontuberculous mycobacteria in persons who have received heart or other solid-organ transplants remain a rare cause of late infectious complications and occur ≈3.5 years after transplantation (*1*,*4*). In the subgroup of heart transplant recipients, skin disease is the most common clinical manifestation, followed by pulmonary and disseminated disease; *M. kansasii, M. avium* complex, and *M. haemophilum* infections are most frequently encountered (*1*,*5*).

M. genavense causes up to 12.8% of all nontuberculous mycobacteria infections in AIDS patients; these infections are clinically similar to those caused by the *M. avium* complex (*1*,*6*).

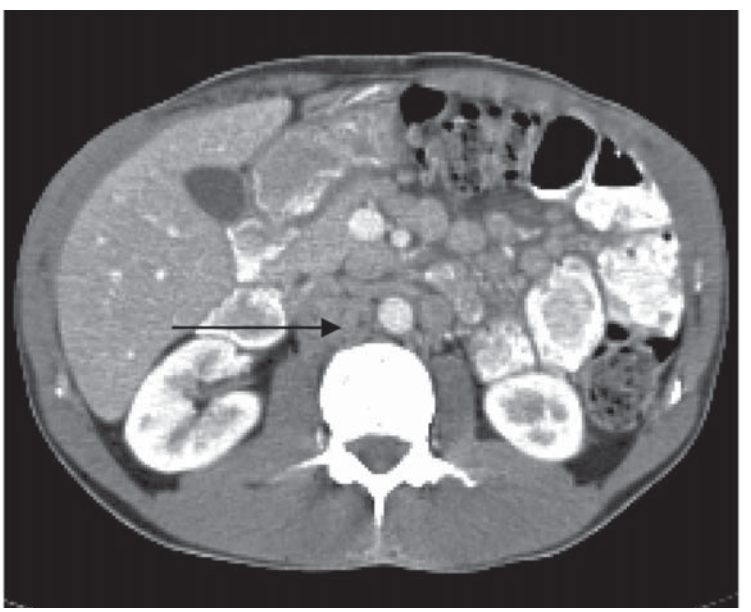

Figure. Initial computed tomographic scan of the abdomen, performed after intravenous injection of contrast dye, showing numerous enlarged para-aortic lymph nodes (arrow).

M. genavense infections occur only rarely in persons other than AIDS patients (as in the present case), but they always occur in immunocompromised persons (*7*,*8*). To date, only 1 case of disseminated infection has been reported in a solid-organ (kidney) transplant recipient; the diagnosis was made by molecular identification in isolates from blood and marrow cultures. That patient died of complications from *M. genavense* infection (*9*). Because *M. genavense* is a fastidious organism, the infections it causes are difficult to diagnose and their frequency is probably underestimated, which may change with increased use of direct molecular biological methods.

Optimal treatment of *M. genavense* infections has not been established (*10*). Experience with *M. genavense* infections in AIDS patients and with other nontuberculous mycobacteria infections in solid-organ transplant recipients suggests that at least 2 antimicrobial drugs should be used for a prolonged period; when possible, immunosuppressive drugs should be concurrently reduced (*1*,*3*,*6*,*10*). Outcome of nontuberculous mycobacteria infections in transplant patients is highly variable (*1*,*5*) but was satisfactory in the present patient, who was treated with quintuple antimicrobial-drug therapy and reduced immunosuppressive therapy.

This case of a disseminated infection due to *M. genavense* in a heart transplant recipient was diagnosed early. Universal 16S rRNA gene sequencing after amplification directly from intestinal biopsy specimens enabled fast diagnosis and appropriate management.

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Isolation of Novel Adenovirus from Fruit Bat (*Pteropus dasymallus yayeyamae*)

To the Editor: Bats are thought to be one of the most important reservoirs for viruses such as Nipah virus, severe acute respiratory syndrome (SARS) coronavirus, and Ebola virus (*1*). These pathogens became known after extensive surveys of bats following outbreaks. As a first step in investigating unidentified pathogens in bats and to help forecast the potential threat of emerging infectious diseases, we tried to isolate and characterize viruses that persistently infect bats. In the process, we isolated a novel adenovirus from a fruit bat in Japan.

Pteropus dasymallus yayeyamae, or Ryukyu flying fox, is a fruit bat of Japan. With the permission of the governor of Okinawa, we caught 1 adult male bat of this species and used its spleen and kidneys to establish primary cell cultures. On the 4th passage of the primary adherent cells derived from the spleen, a cytopathic effect (CPE) appeared without any visible

microbe, indicating that the cell culture contained a virus. The virus, tentatively named Ryukyu virus 1 (RV1), caused apparent CPE on primary kidney cells derived from a Ryukyu flying fox and on our established bat kidney T1 (BKT1) cells, which were derived from the kidney of a horseshoe bat (*Rhinolophus ferrumequinum*) and transformed with expression plasmid DNA encoding the large T antigen of replication origin-defective simian virus 40.

To identify the virus, RV1, we applied the rapid determination of viral RNA (RDV) system version 1.0 (*2*). However, no viral nucleic acid sequence was detected from an RNA sample in the RV1-infected BKT1 cells. For detection of viral DNA, we developed a system for rapid determination of viral DNA sequences (RDV-D) by minor modification to the RDV system for RNA viruses (*2–4*). The results indicated that 2 of the fragments were homologous to the gene encoding the precursor of terminal protein (pTP) of adenoviruses. Further RDV-D analysis showed that 6 fragments (139 bp, DDBJ/EMBL/GenBank accession no. AB302970) were homologous to the pTP gene and that another 6 fragments (316bp, DDBJ/EMBL/GenBank accession no. AB302971) were homologous to the gene encoding the precursor of protein VI (pVI) of adenoviruses. These results indicated that RV1 must belong to the family *Adenoviridae*.

To further confirm that RV1 isolate was an adenovirus, we used PCR and sequencing. We performed the first reaction with the outer primer pair (polFouter and polRouter) of a nested PCR method, targeting the viral DNA polymerase gene with highly degenerate consensus primers that have been described recently (*5*). A fragment of ≈550 bp was amplified from RV1 as well as from human adenoviruses-1, -3, -4, and -7 (data not shown). Sequence analysis of the amplified product (DDBJ/EMBL/GenBank accession no. AB303301) showed that RV1 was homologous to tree shrew adenovirus 1 (70.0% amino acid sequence identity), porcine adenovirus 5 (69.2%), canine adenovirus 1 (68.9%), human adenoviruses-3, -16, -21 and -50 (68.9%), and other viruses (>64.8%) in genus *Mastadenovirus*, but less homologous (46.7%–57.8%) to viruses in other genuses, *Siadenovirus, Aviadenovirus,* and *Atadenovirus*. In addition, a phylogenic tree based on amino acid sequences indicated that RV1 belongs to family *Adenoviridae*, genus *Mastadenovirus* (Figure).

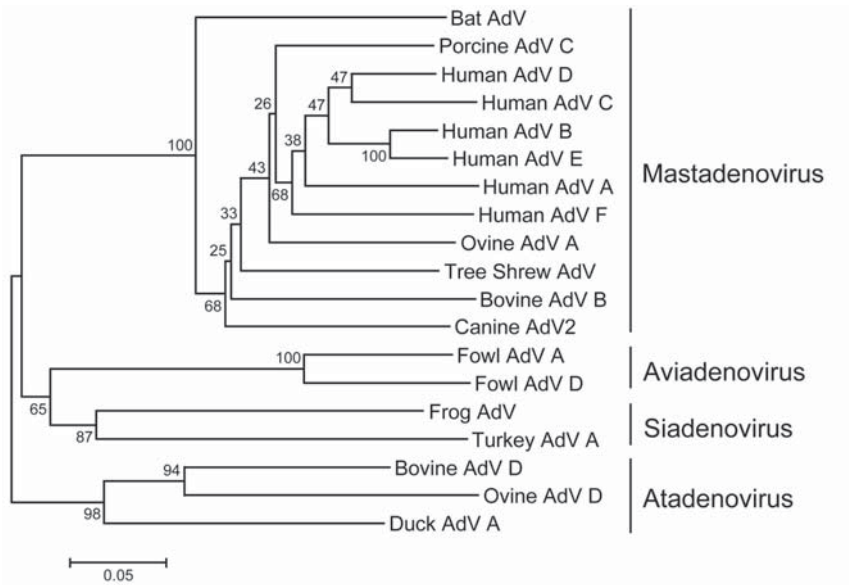

Figure. Phylogeny of adenoviruses based on analysis of partial amino acid sequences of DNA polymerase protein. Trees were estimated by using the neighbor-joining method based on the amino acid pairwise distance and MEGA 4.0 software (www.megasoftware.net). Numbers represent percentage bootstrap support (100 replicates).

Electron microscopy of RV1-infected BKT1 cells indicated that RV1 accumulated in the nucleus and that the size of capsids was 60–70 nm (data not shown). Restriction endonuclease analysis of the RV1 genome indicated that the genome was ≈20–30 kbp (data not shown). These features are consistent with RV1 being an adenovirus.

Until now, a number of RNA viruses have been isolated from bats, but isolation of DNA virus is rare (*1*). The isolation of the novel adenovirus seems to be possible because of usage of the primary cells originated from the host; DNA viruses might have more restricted host range than RNA viruses and require host-originated cells for the growth. In addition, our success in DNA virus isolation might have resulted from usage of the adult animal latently and persistently infected with DNA viruses such as adenovirus and herpesvirus.

In conclusion, we isolated a novel virus from a fruit bat. This virus was isolated from a healthy bat, which suggests that the virus may persistently infect fruit bats. Although its pathogenicity for humans is still unknown, knowledge of RV1 will be useful in epidemiologic studies of infectious diseases emerging from bats because persistently infecting viruses might be isolated together with primary pathogens. We are planning to establish cell lines from bats and isolate more viruses from persistently infected bats.

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Fluoroquinolone-Resistant Group B Streptococci in Acute Exacerbation of Chronic Bronchitis

To the Editor: Fluoroquinolones (FQs) that are active against streptococcal species (e.g., levofloxacin and moxifloxacin) have been recommended by numerous national health authorities and international organizations for treating acute exacerbations of chronic bronchitis and pneumonia in adults (*1*). However, use of these antimicrobial drugs for treating community-acquired infections has led to an increase in FQ-resistant strains in bacteria such as *Streptococcus pneumoniae*. Group B streptococci (GBS, e.g., *S. agalactiae*) are the leading cause of invasive infections (pneumonia, septicemia, and meningitis) in neonates. GBS are also associated with bacteremia, endocarditis, and arthritis, and are responsible for deaths and illness in nonpregnant women with underlying diseases and in elderly adults (*2*). We describe, to our knowledge, the first GBS clinical isolate in France resistant to FQ; the isolate was from a patient treated with levofloxacin.

GBS CNR0717 strain was isolated as the predominant bacterium in a culture (>10^7 CFU/mL) from 2 purulent sputum samples from an 80-year-old man (leukocytes >25, epithelial cells <10) obtained 8 days apart. This patient was treated for 2 weeks with levofloxacin, 750 mg/day, for acute exacerbation of chronic bronchitis. No other relevant respiratory bacterial pathogens were present in these samples. GBS CNR0717, a capsular serotype IV strain, was suspected to have reduced susceptibility to FQs because no inhibition zone was observed around disks containing norfloxacin and pefloxacin disks, and reduced diameters were observed around disks containing ciprofloxacin and levofloxacin. Antibiograms were performed according to recommendations of the Clinical and Laboratory Standards Institute (*3*) on Mueller Hinton agar (Bio-Rad, Marnes la Coquette, France) supplemented with 5% horse blood. This strain was susceptible to all other antimicrobial drugs usually active against GBS (penicillin, erythromycin, clindamycin, tetracycline, rifampicin, vancomycin) and showed low-level resistance against aminoglycosides. MICs for 6 FQs (Table) indicate that GBS CNR0717 was highly resistant to pefloxacin and norfloxacin, with MICs >64 mg/L, and showed increased MICs for ciprofloxacin, sparfloxacin, levofloxacin, and moxifloxacin. No reduction of FQ MICs was observed with reserpine (10 mg/L), which indicated that resistance to FQ was not caused by an active efflux pump system.

Three major mutations have been reported for FQ resistance in streptococci at codon positions 81 in *gyrA* and 79 or 83 in *parC* (*4*). DNA sequence analysis of these regions showed a mutation in *parC* (Ser 79 → Tyr) but not in the wild-type susceptible strain (NEM316). No mutation was detected in the *gyrA* gene. FQ resistance in streptococci is acquired through a stepwise process and has been extensively studied in *S. pneumoniae*. First-step mutants conferring low-level resistance generally result from mutations in either *gyrA* or *parC*. There is also

Table. MICs of fluoroquinolones for strains of group B streptococci (GBS), France

	MIC (mg/L)*					
Strain	Pef	Nor	Cip	Spa	Lev	Mox
GBS CNR07017	>64	>64	4	1	4	1
GBS NEM316	16	8	2	0.5	1	0.25

*Pef, pefloxacin; Nor, norfloxacin; Cip, ciprofloxacin; Spa, sparfloxacin; Lev, levofloxacin; Mox, moxifloxacin.

a molecule-dependent target specificity: mutations in *parC* are generally selected by pefloxacin, ciprofloxacin, and levofloxacin, and those in *gyrA* are selected by sparfloxacin, gatifloxacin, moxifloxacin, gemifloxacin, and garenoxacin (*5*). In second-step mutants, mutations are present in both *parC* and *gyrA* and confer resistance to the antistreptococcal FQs levofloxacin, moxifloxacin, and gatifloxacin.

FQ resistance in GBS has been reported in Japan, the United States, and Spain (*6*–*8*). Up to now, all FQ-resistant GBS strains described were highly resistant because of point mutations in *gyrA* and *parC* QRDR; a *parC* mutation at position 79 was present in all strains. These strains were isolated from elderly adults who, in some cases, had received quinolone therapy. Low-level resistance to FQ in GBS CNR0717 was associated with a Ser 79 → Tyr mutation in *parC*. Therefore, although the FQ sensitivity of this strain is unknown, a first-step mutant could have been selected in vivo as our patient was treated with levofloxacin for 2 weeks.

GBS is an unusual cause of acute bacterial exacerbation of chronic bronchitis compared with other respiratory pathogens such as *S. pneumoniae,* but pathologies associated with this bacterium are changing. Clinical microbiologists should be aware of these changes and test isolates of *Streptococcus* spp. for susceptibility to FQs. This report indicates that FQ resistance among streptococci is a growing concern and that levofloxacin can select in vivo *parC* first-step mutants that will facilitate emergence of high-level FQ-resistant GBS strains, as demonstrated in vitro for *S. pneumoniae* (*9*). Finally, although FQ treatment is recommended for high-risk groups with acute exacerbations of chronic bronchitis, these antimicrobial drugs must be reserved for situations in which there are no effective alternative drugs to treat infections caused by multidrug-resistant bacteria. For susceptible strains, β-lactams, which still constitute the first-line recommended antimicrobial drugs, should be used for treatment of these patients (*10*).

This study was supported by the Assistance Publique Hôpitaux de Paris, Institut de Veille Sanitaire, Institut National de la Santé de la Recherche Médicale, Centre National de la Recherche Scientifique, and Université Paris Descartes.

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Dengue and Relative Bradycardia

To the Editor: In a recent letter to Emerging Infectious Diseases, Lateef and colleagues identified a relationship between dengue and relative bradycardia in patients in Singapore. They stated that "To our knowledge, this sign has not been previously associated with dengue" (*1*). Unfortunately, the association of dengue fever with relative bradycardia has already been well established and is certainly not a new finding (*2*,*3*). Despite this, however, there is no harm done in reinforcing an often forgotten clinical sign that can assist in the diagnosis of dengue, especially in those countries with limited resources.

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Importation of Poliomyelitis by Travelers

To the Editor: In July 2007, an Australian traveler imported polio from Pakistan to Australia (*1*). He was a 22-year-old man who had immigrated to Australia and had traveled to his country of origin (Pakistan) to visit friends and relatives. Pakistan is one of 4 countries (Afghanistan, India, Nigeria, Pakistan) where polio is still endemic. A diagnosis of polio was made shortly after his return to Australia. Australia was certified as polio-free in 2000. Australia will not be the last industrialized country affected by importation of polio. All countries are at risk until polio has been completely eradicated.

Between 2003 and 2006, polio was imported by travelers (e.g., refugees, pilgrims, traders) to 24 polio-free countries (*2*). The origin of these importations was largely the 4 countries where polio transmission was never completely interrupted. The importations resulted in about 1,400 secondary cases (*2*). The resurgence of polio by international spread was a setback to the Global Polio Eradication Initiative that had successfully decreased the number of polio-affected countries to only 9 in 2002.

The revised International Health Regulations, IHR (2005) (*3*), entered into legal force on June 15, 2007. These regulations provide the legal framework for coordination of the international effort to reduce or prevent international spread of diseases of public health concern. IHR (2005) (*2*) lists polio as one of the diseases of public health emergencies of international concern. Preventing importation of polio into polio-free countries is therefore a test case for the revised International Health Regulations (*4*). Compared to the previous IHR (1969), IHR (2005) has moved away from the definition of fixed maximum measures relating to specific diseases and instead focuses on the issuance of context-specific recommendations, made either on a temporary emergency basis (a temporary recommendation) or routinely for established ongoing risks of disease spread (a standing recommendation).

One strategy to protect polio-free countries from reintroduction of wild poliovirus is by requiring proof of polio vaccination for all incoming travelers from polio-endemic countries. This was proposed by the Advisory Committee on Poliomyelitis Eradication in October 2006. The rationale is similar to that used for yellow fever, currently the only disease for which proof of vaccination may be required for travelers as a condition of entry to a country. The proposal of the Advisory Committee of Poliomyelitis Eradication was discussed at the World Health Assembly in May 2007 (*5*). Although the main strategy for polio eradication continues to be attaining high vaccination coverage against polio in all countries, the 193 member states have also adopted the resolution to "continue to examine and disseminate measures that member states can take for reducing the risk and consequences of international spread of polioviruses, including, if and when needed, the consideration of Temporary or Standing Recommendations, under the International Health Regulations (2005)" (*3*).

The recent polio importation by an inadequately vaccinated traveler would add impetus to such considerations. However, this case also shows that focusing on travelers from polio-endemic countries alone may not be sufficient. Immigrants from developing countries to industrialized countries who subsequently return to their home countries to visit friends and relatives may also be at increased risk if traveling to polio-endemic countries, in particular as many may not have received adequate childhood vaccination including vaccination against polio (*6*). Targeting those visiting friends and relatives is therefore a potential additional strategy to reduce the risk for the worldwide spread of polio.

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In Response: After poliomyelitis was imported into Australia, Wilder-Smith and colleagues (*1*) call for proof of vaccination for travelers from polio-endemic countries. Although superficially attractive, their recommendation won't be extremely effective, will be burdensome for polio-endemic and polio-free countries, and is unnecessary.

Documenting vaccination may slightly reduce, but will not eliminate, importations. Vaccination simply does not provide high-level protection against poliovirus infection. Children recently vaccinated with either oral poliomyelitis virus (OPV) or inactivated poliomyelitis virus (IPV) shed poliovirus following a challenge OPV dose (*2*). Because secretory immunity falls rapidly, a high percentage of persons vaccinated years or even decades ago will become transiently infected when exposed to poliovirus and will excrete virus for weeks. Lower vaccine efficacy in developing countries (*3*) further compounds the issue.

Screening programs are likely to be costly and will not be simple to implement. Unanswered questions include the following: Is a single dose of IPV or OPV immediately before departure adequate? Are boosters needed? Why not include countries with imported wild or vaccine-derived poliovirus (VDPV) outbreaks? Can polio-free areas of polio-endemic countries (e.g., Kerala) be exempted? Must records be certified? Can fraudulent vaccinations be detected or prevented?

Data on importations clarifies any need for requiring vaccination of travelers entering polio-free countries. Polioviruses are imported regularly, yet outbreaks are rare. The Australian case comprised 1 imported case. Similarly, no paralytic cases followed the recent importation of a poliovirus from Chad into Switzerland (*4*) or the 2005 Minnesota VDPV infections (*5*). The United Kingdom has been polio-free for decades despite close ties with India, Pakistan, and Nigeria. Polio outbreaks (both wild and VDPV) occur where immunization coverage is low. The last major outbreak in Western Europe occurred in a Dutch religious group that refuses immunization. The 2005–06 global outbreak affected polio-free countries where polio immunization coverage had fallen after transmission was interrupted.

Countries at risk for polio importation because of low vaccination coverage should focus on improving their immunization programs, not vaccinating and screening travelers. Australia and other polio-free countries can best protect themselves against importations by supporting eradication efforts in polio-endemic countries.

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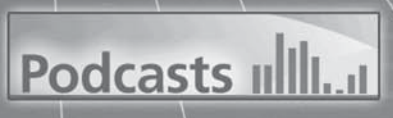

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Handbook of Zoonoses: Identification and Prevention

Joann Colville and David L. Berryhill

Mosby, St. Louis, Missouri, USA, 2007

ISBN: 9780323044783
Pages: 272; Price: US $44.95

In "Handbook of Zoonoses: Identification and Prevention," authors Joann Colville and David L. Berryhill laud their book as an ideal reference for veterinarians, veterinary technicians, and professional students, and as a general resource for healthcare professionals to help them understand and manage zoonotic diseases. Information on common, and currently topical, zoonoses are included; the book addresses diseases caused by bacteria, viruses, parasites, fungi, and prions. For each disease, a common set of concepts are covered: the degree of illness and death associated with the disease; the etiology, hosts, and routes of transmission; a brief description of the disease manifestation in various animal species and in humans; general guidance for treatment in both animals and humans; and recommendations for prevention. Although the authors present some information on diseases that occur outside the United States, this handbook focuses more on diseases in the United States that readers may come into contact with or hear about on the news.

The terminology used to cover zoonotic diseases may be useful to healthcare professionals because basic information is provided in easily understandable language for lay patients and clients. As an actual source of information for healthcare professionals, however, this handbook is not as useful as other available texts. The book covers aspects of many of these diseases somewhat superficially and makes generalizations that could be misleading when examined in greater depth. This weakness is exacerbated by the fact that no references are provided, either as source data for citation or as pointers for readers looking for more information on a particular disease. Given the lack of overall detail in the handbook, this is a significant shortcoming. And, while an overall goal of the book is to provide information to help healthcare professionals manage zoonotic diseases, the authors do not present specific treatment guidance or common differential diagnoses for the diseases covered. Instead, the authors provide the occasional piece of trivia about certain diseases covered in the handbook that a lay reader may find engaging—that Ted Nugent wrote a song called "Cat Scratch Fever" and that rumors exist of a "Hollywood Tapeworm Diet." Overall, this handbook may be best suited for the lay person who has an interest in zoonotic diseases and some preexisting knowledge of disease transmission and pathogenesis.

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The Microbiology Bench Companion

J. Michael Miller

ASM Press, Washington, DC, USA, 2007

ISBN-10: 1555814026, ISBN-13: 978-1555814021
Pages: 120; Price: US $36.90

Diagnostic testing for infectious diseases is an increasingly complex area of laboratory medicine. The microbial community continues to evolve and adapt to changing environmental influences, and the distribution of human pathogens has become more global. Our recognition of the spectrum of microorganisms that cause invasive human disease has exploded with the use of culture-independent methods to detect and characterize pathogens. Clinicians, epidemiologists, and public health officials can benefit from consultative interactions with laboratory professionals to assist with optimizing diagnostic test options and interpretation of test results. For technologists without access to board-certified laboratory professionals who can guide the work-up of infectious agents in a microbiology laboratory, a concise guide can be extremely beneficial.

In this handbook, J. Michael Miller, a highly experienced clinical microbiologist, distills a great deal of information into 120 pages, largely formatted as tables and flowcharts. Molecular methods for detecting or identifying microorganisms are notably absent, which may reflect the author's intent to address readers who perform conventional diagnostic tests only. The handbook is divided into 3 sections. Section 1 focuses on routine laboratory bench algorithms for identifying bacteria, fungi, and parasites. Although most of this information can be found in clinical microbiology textbooks, the flowcharts are an easy reference, especially for medical technologists. Section 2 is entirely formatted

with tables listing clinical syndromes (e.g., cellulitis, pneumonia, gastroenteritis) and their possible infectious causes. The lists of etiologic agents are extensive, yet practical, and would be of most benefit to infection control practitioners, nurses, and laboratory technologists. Section 3 is devoted to therapeutic choices for various microorganisms and is intended to inform laboratorians of treatment options; it is not a guide for therapeutic decision-making by clinicians. Although certain taxonomic classifications and susceptibility guidelines do not reflect the current standard, most information, such as annotated remarks for pathogens classified as select agents, is timely.

The author valiantly furnishes us with pearls and nuances of clinical microbiology in this clear and concisely written handbook. The task was Herculean. As the author aptly notes in the introduction, no handbook can capture every parameter or indication for identification of all clinically relevant microorganisms and describe these microorganisms in public health or patient-centered contexts. Overall, the author delivers a wealth of information that can benefit technologists and healthcare practitioners in regions with limited access to professionals who specialize in clinical microbiology or infectious diseases.

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Bioviolence: Preventing Biological Terror and Crime

Barry Kellman

Cambridge University Press, Cambridge, United Kingdom, 2007

ISBN-10: 0521709695, ISBN-013: 978-0521709699
Pages: 392; Price US $28.99

Even before the anthrax attacks in 2001, public health agencies and partner sectors had begun intensifying efforts to detect and respond to the specter of biologic agents used as instruments of terror. The events in 2001 highlighted the substantial preparedness gaps and needs in multiple dimensions, particularly the requirements for coordinating the work of public health and law enforcement, sectors that operate under different jurisdictional configurations and legal regimes. This book is written by a law professor who begins by positing the thesis that humanity is vulnerable to bioterrorism because current international legal regimes are inadequate to support preventive policies. The author may thus be overly ambitious by attempting to cover this topic on a global scale, rather than through the prism of 1 or a few governance systems.

This book may be particularly helpful to persons who want to learn more about basic concepts regarding the methods of bioterrorism. For example, the second chapter provides an overview and description of biologic agents identified as candidates for use by terrorists, and the third chapter presents a synopsis of historical milestones in the use of bioweapons. The second part of the book offers a conceptual treatment of the author's beliefs about factors accounting for the global failure to effectively confront the threat of biologic agents by multiple actors, and combines this with a focused discussion of 4 categories of measures to reduce bioterrorism. These categories are interdiction (a practically framed summary), denial of access to methods of bioterrorism, preparedness (i.e., detection and response), and nonproliferation regimens. The author concludes with a call for the establishment of "a global governance architecture for preventing bioviolence."

The book's utility for practical applications seems constrained, in part, by a limitation common to single-authored books on topics with myriad and complex technical dimensions. In particular, examining bioterrorism must take into account the convergence of numerous and complex fields, including forensic and laboratory sciences, public health, law enforcement, and behavioral sciences, to name only a few. In addition, although some chapters provide information helpful for shaping readers' understanding of particular issues, in many instances the text falls short of being practically relevant. For example, within the chapter on public health preparedness, the author devotes only 3 paragraphs to the critically important issue of "law enforcement–public health cooperation," which, since the 2001 anthrax attacks, has been the focus of several major initiatives within the United States.

An additional point is that the author appears to be coining a new term, *bioviolence* ("...the infliction of harm by the intentional manipulation of living micro-organisms or their natural products for hostile purposes"), for which he also provides a rationale. Yet to be determined is whether this term truly is helpful or possibly confusing because of the already well-established lexicon and conceptions surrounding bioterrorism. On balance, however, this book can be recommended because it helps to address a void in the literature, particularly in relation to concepts of preventing bioterrorism, and because it represents another step

toward establishing the multidimensional knowledge base necessary to enhance preparedness.

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Encyclopedia of Infectious Diseases: Modern Methodologies

Michel Tibayrenc, editor

Wiley & Sons, Inc., Hoboken, New Jersey, USA, 2007

ISBN: 978-0-471-65732-3
Pages: 747; Price: US $175.00

Michel Tibayrenc is someone with big ideas. For many years, he has championed a vision of multidisciplinary systems to approach infectious diseases and public health. One of his longstanding ideas has been to develop a global network of regional institutions built on the model of the US Centers for Disease Control and Prevention. To address routine and emerging disease challenges, this network would blend state-of-the-art molecular approaches such as evolutionary genetics, proteomics, sequencing, and subtyping with traditional field investigations and surveillance.

In support of this vision, Dr. Tibayrenc has edited a book entitled Encyclopedia of Infectious Diseases: Modern Methodologies. The dictionary defines an encyclopedia as "a work that contains information on all branches of knowledge or treats comprehensively a particular branch of knowledge, in articles usually arranged alphabetically by subject" (*1*). When asked to review the book, I was therefore curious as to how he would cover such a broad topic.

Despite its lengthy 747 pages, this book is not an encyclopedia of infectious diseases. First, the content has no obvious pattern, alphabetical or otherwise. As an example, the opening chapter is "Pulmonary Tuberculosis and *Mycobacterium tuberculosis*: Modern Molecular Epidemiology and Perspectives." Four chapters later, a somewhat redundant chapter called "Molecular or Immunological Tools for Efficient Control of Tuberculosis" appears. In between are unrelated chapters on livestock diseases, HIV/AIDS molecular epidemiology, and uncultured pathogens; these are followed by chapters on leishmaniasis and epidemics of plant diseases.

Second, the book is hardly comprehensive or consistent. It contains full chapters on leishmaniasis, severe acute respiratory syndrome, cholera, hantavirus infection, and Chagas disease, and 2 chapters each on tuberculosis and malaria. Some of these chapters are relatively straightforward reviews; others use the disease for illustrative purposes only. The chapter on livestock diseases has 18 references; the one on leishmaniasis, 402. An important pathogen like *Staphylococcus aureus* is virtually unmentioned; *Streptococcus pneumoniae* does not even appear in the index.

So if the book isn't an encyclopedia, what is it? The best description would be an interesting potpourri of essays on various aspects of infectious diseases. One chapter is even entitled, "Topical Debates." Although the emphasis is on pathogen differentiation and evolution, the content runs the gamut from mathematical modeling to geographic information systems to remote sensing to morphometrics. The book even contains a fascinating chapter devoted to archeological epidemiology (mummies) and a whopping 61-page chapter on infectious diseases and the arts, including an extensive list of movies with infectious disease themes.

This assessment by no means trivializes the book. Many of its chapters are extremely well written and do a wonderful job of distilling complex concepts into narrative that even a novice infectious disease scholar could understand. Particularly fine examples are the chapters on influenza evolution and on geographic information systems.

So who would benefit from this book? Not those engaged in clinical medicine and those looking for a practical encyclopedia of infectious diseases; they will be disappointed. This book is fundamentally a loving and personal testament to Dr. Tibayrenc's vision of multisystems approaches to emerging diseases. Those who share this vision will find a great deal to value in this text.

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Books, Other Media.

Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Emerging Infectious Diseases: Trends and Issues, 2nd Edition

Felissa R. Lashley and Jerry D. Durham, editors

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ISBN: 0826102506
Pages: 591; Price: US $70.00

This second edition of Emerging Infectious Diseases: Trends and Issues, edited by F.R. Lashley and J.D. Durham, appearing 5 years after the first edition, has added several important diseases such as severe acute respiratory syndrome, avian influenza, and monkeypox. The authors also have updated the chapters included in the first edition. At nearly 600 pages, the book gives a broad overview of 22 emerging diseases with mention of many others. Topics such as antimicrobial drug resistance, bioterrorism, and travel-associated issues also are covered. Persons interested in having a bedside companion about microbes out there in the world waiting to infect humans will enjoy this book, and medical personnel should find it useful. Many chapters begin with a clinical case history, which captures the reader's attention and transports him or her into the mindset of a clinician. Yet the book by no means can serve as a diagnostic manual. Rather, it provides basic information on diseases and issues that should be kept in mind in diagnostics, such as behavioral, cultural, and environmental factors that influence the likelihood of infection with particular pathogens.

The broad scope of topics covered is impressive, but the book does not cover any specific disease rigorously. To avoid inaccuracies, the submitted manuscript would have benefited from review by scientists with current expertise regarding specific pathogens. For instance, it is stated that West Nile virus–infected horses maintain "moderate to high levels of virus," whereas recent research indicates low transient viremia in equines that cannot infect mosquitoes (*1*). In addition, some statements may be misleading, such as "high levels of the [West Nile] virus in birds may persist for long periods of time (20–100 days); thus, migratory birds are implicated in the introduction of WNV." Recent studies indicate that high levels of viral RNA but low levels of infectious virus have been recovered from birds up to 6 weeks after infection (*2*,*3*). The epidemiologic significance of these findings is not clear.

Additionally, other concepts are poorly explained, e.g., the discussions on dengue secondary infections and authochthonous transmission. Broad, sweeping statements are made, which by their nature are inaccurate, such as the statement in the introduction that Ebola "came and disappeared." If only that were so! Also, some important emerging/reemerging diseases are not covered in detail, such as chikungunya, or at all, such as Japanese encephalitis. More problems occur with the glossary, which, for example, defines an arthropod as a "vector belonging to the phylum Arthropoda that transmits an organism from 1 host to another." This is more a definition of an "arthropod vector" since not all arthropods are vectors. Therefore, scientists, especially those involved in public health, may not find their particular areas of expertise described satisfactorily, but they will find in many areas outside their immediate field of knowledge a useful compact description that addresses the highlights of a particular disease.

Putting these complaints aside, the book serves well as a point of introduction to the major diseases described. The bibliographies have been strengthened since the first edition and are useful guides for further in-depth inquiry. The 4 appendixes are a strong point. Appendix D provides information on available resources for learning more about specific diseases, appendixes A and B list and describe emerging/reemerging infectious diseases by organism and by modes of transmission, and appendix C lists measures to prevent these diseases. Overall, we recommend this book to anyone who wants an introduction to the public health aspects of emerging diseases.

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This Time of Dying

Reina James

Portobello Books Ltd., London, United Kingdom, 2006

ISBN: 9781846270468
Pages: 296; Price: US $14.55

A must-read for those who think flu pandemic preparedness is much ado about nothing, this first novel by Reina James brings home the severity and plausibility of a flu disaster. And without data, statistics, or comment, it makes a compelling case for progress on the matter of influenza, to ease suffering, prevent spread, and possibly eliminate the scourge.

A series of journal entries beginning October 14 and ending November 7, 1918, this story of humans under stress unfolds in London against the savage backdrop of World War I and amidst the oft-recorded pestilence of Spanish flu. Without speculation, the author constructs population drama, one patient and one family at a time. The reader is drawn into it instantly and stays absorbed 'til the end.

Characters from all walks of life make up the cast of the unfolding tragedy. They chase about in their masks obsessing over work, social and self-imposed restrictions, unrelenting class barriers, and daily trivia until the schools close, the servants die or flee the ailing households, businesses fail, or until they succumb, filling the streets, mortuaries, graveyards, churches. We are allowed into their disinfected homes, to watch the banality, madness, or sheer horror of their lives.

Some are stars. The heroic physician is back from the war with heightened awareness of the health emergency. "A plague is now among us which may well leave the earth to the animals," he writes in a note intended to alert the health authorities. "You must stop the movement of troops, close our ports and warn others to follow suit." He dies, "a blue man in the road," before he can deliver the message. "His lips and ears were purple-blue, like a plum, and the skin on his face was mottled and pale but still tinged with blue, as if he'd been wiped with an inky rag."

The undertaker, who finds the warning note crumbled in the dead physician's palm, is a man who plays the piano in between constructing coffins and practicing the family trade. He becomes increasingly unsettled by the note as the dead overwhelm his business, his city, his life. "The world's body can hardly draw breath; it is sick and brought to its bed," the physician had written. "Its wounds are open. The young spill out ... Death is crossing every sea." He confirms that indeed the dying "were unusually young," tries in vain to inform the authorities, and concludes with disbelief that "They had no plan." The knowledge imparted by the note changes the undertaker's life, which is already complicated by his universally disapproved affair with a woman "a little bit above his station."

His friend dismissed her, "She's like a bloke." Her friend, in a moment of weakness, thought her "a foolish ageing woman with a weak, offended face. Her lips merged unpleasantly into the skin surrounding them; her cheeks were dragged flat by failing muscle. There was too much eyelid and too little eyebrow. There was even a suggestion of fluff above the mouth, of the type that would inevitably grow into a moustache." Their attraction, slowed by awkwardness and saved by spunk, adds an endearing almost hopeful quality to a tale rife with decomposing bodies.

Another physician, a retired practitioner, is exhausted by the torrent of patients and his inability to improve their situation. Asked his opinion about the epidemic, he retorted angrily, "I haven't got an *opinion* ... I'm too busy." Exasperated by people reporting "cyanosis," he admitted that few things irritated him "more than patients using medical terminology." In the absence of medical interventions, he suggested "fresh air in the bedroom, aspirin for headache, plenty of fluids and light food only, if tolerated." His unloved wife and assistant by default props him up in bed to sign a few more death certificates before he collapses.

Dedicated by the author to her maternal grandparents who died of it, this account of Spanish flu in London rings true for its hold on human behavior and of course for the flu, whose specter looms on our horizon. Along with the science, along with the vaccine, a dose of past history in human terms warns against underestimating a new pandemic. As James put it, "if every one of the newly bereaved were to hold a lantern in the sky, the man in the moon would think the world to be on fire."

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A Rondelay (Without Cadenza) By The Virion Of Influenza

Edwin D. Kilbourne

Now you have me—sucrose-banded,
Enveloped and negative-stranded
Spiked and cleaved and slightly dented
Into pieces eight, segmented—
Without mercy I've been strained
Through filters—then been bromelained, and
Torn apart by each detergent
With a haste unseemly, urgent—
All my helices displayed
Just to show you how I'm made.
My polypeptides have been mapped
My hemagglutinin unwrapped—
All my sequences are clear—
All the way from Arg to Ser.
With techniques sharp and newly honed
My very genes have now been cloned—
Transplanted to an alien host
Wherein my evanescent ghost
As solitary as an elf
Has managed to express myself.
And scientists have labored nights
To probe my antigenic sites
Some fresh from school with new diplomas
(Aided by their hybridomas)
Scramble up trimeric slopes
Counting all my epitopes
And every Ph.D. or pupil
Utterly devoid of scruple
Mates me with complete abandon—
Asks, then, why my genes are random
Can I kiss and never tell
When genotyped upon a gel?
Which, if inspected with acuity
Will document my promiscuity—
Must you write, in fat reports
How flagrantly I reassort?
No boundaries have my misbehavin'
Horsey set or duck or avian—
In other moments less sublime
You've put my perils before swine!
And yet in 1981
Despite the work that has been done
The epidemics come and go
As regular as winter snow.
And people cough and people die
And all of you still wonder why.
I'm so perverse and ever mutable
And so eternally unscrutable.
But think about just what you'd do
If there were really
No more flu!

Courtesy of the Author. Published in Genetic Variation among Influenza Viruses. DP Nayak, editor. New York: Academic Press, Inc.; 1981.

Dr Kilbourne has spent most of his professional lifetime, beginning in 1948, on the study of influenza. During his tenure at five medical institutions, he has contributed particularly to discoveries that have facilitated vaccine development. As an avocation, he has published light verse and essays that have appeared in a number of nonmedical periodicals, including Saturday Review; some of his works have been collected recently in book form in Strategies of Sex. He is a member of the National Academy of Sciences, the Association of American Physicians, and the American Philosophical Society, among other honorary societies. He may be contacted by email: ekilbourne@snet.net

Henry Ossawa Tanner (1859–1937). The Banjo Lesson (1893). Oil on canvas (124.46 cm × 90.17 cm). Hampton University Museum, Hampton, Virginia, USA

Artistic Light and Capturing the Immeasurable

Polyxeni Potter*

"After school, I would often go down on Chestnut Street to see the pictures in Earle's Galleries," Henry Ossawa Tanner recalled about his early years (*1*). He was especially drawn to the marine subjects of T. Alexander Harrison (1853–1930). "After drinking my fill of these art wonders, I would hurry home and paint what I had seen, and what fun it was" (*1*). When at age 12 or 13, he saw for the first time an artist at work in Philadelphia's Fairmount Park, he knew his life's calling.

Tanner was born in Pittsburgh but raised in Philadelphia, the oldest child in a large activist family. His mother, a former slave, escaped through the Underground Railroad. His father was Bishop of the African Methodist Episcopal Church, a denomination formed to protest not dogma but dehumanization and barriers to spiritual expression. "I have no doubt an inheritance of religious feeling," the artist wrote (*1*). His middle name was derived from Osawatomie, the town in Kansas where abolitionist John Brown started his antislavery campaign.

As often the case with budding artists, the pressure was on Tanner to become successful in a conventional line of work. Nevertheless, he enrolled in the Pennsylvania Academy of Fine Arts, where he studied with Thomas Eakins (*2*). "Get it, get it better, or get it worse," urged the tough taskmaster and later lifelong friend, "No middle ground of compromise" (*3*). Rigorous training at the academy refined Tanner's skills as a realist painter. Intolerance cued him in to social realities: "Then he began to assert himself and ... one night his easel was carried out into the middle of Broad Street and, though not painfully crucified, he was firmly tied to it and left there" (*4*).

In 1888, he moved to Atlanta, Georgia, where he opened a photography studio and taught drawing at Clark University. He ventured to nearby North Carolina to photograph and paint the countryside and local folk in their daily activities. But, like most of his contemporaries, Tanner longed to travel abroad to study and experience artistic freedom. With the help of early supporters and patrons, he was able to travel to Europe.

He fell in love with Paris and the "helpful influences" that surrounded him there (*5*). He enrolled in the Académie Julian and studied with Jean-Joseph Benjamin-Constant and Jean-Paul Laurens. "In Paris…no one regards me curiously. I am simply 'M. Tanner, an American artist'" (*6*). His life there as an expatriate was interrupted only briefly, when a life-threatening bout of typhoid fever sent him back to Philadelphia; then again, when during World War I he moved to England.

Tanner's work, initially branded by Eakins' meticulous clarity, gradually acquired its own depth and character. Though mainly in the academic tradition, it incorporated elements of symbolism and impressionism. He was alert

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and open to new ideas but only as they aligned with his own search for aesthetic and artistic truth. He rejected prescribed alliances in art as much as in his personal life, "I paint the things I see and believe" (*1*). He refused to be categorized or classified.

Light was a central element in Tanner's paintings and came in shades of Caravaggio or contrasts reminiscent of Rembrandt van Rijn. His palette and loose strokes were suggestive of the impressionists. And whereas his early work reflected all-encompassing interests, his later paintings were exclusively devoted to spiritual themes. But, he wrote, "Religious feeling will not atone for poor art, and vice versa" (*1*). He traveled to the Near East, intrigued by the topography and cultures, which he brought into his biblical scenes in hues of olive and clay, enriching their oriental, mystical quality.

Tanner flourished in France. His work, frequently reviewed in the local press and exhibited in the Salon, was purchased for the national collection at the Musée du Luxembourg. He was named Chevalier of the Legion of Honor. While neither countless awards in France and the United States nor international acclaim could advance his financial situation, the spiritual quality and artistic restraint that came to characterize his work inspired a succeeding generation of artists, among them American favorites Hale Woodruff, Jacob Lawrence, Romare Bearden.

The Banjo Lesson, one of the most famous paintings of the period, was inspired by "Uncle Tim's Compromise on Christmas," a short story illustrated by Tanner for Harper's Young People from a photograph he had staged (*7*). "The only thing in the world that the old man held as a personal possession was his old banjo," read the story, so his gift to the child had to be shared. But "It was the one thing the little boy counted on as a precious future property, and often, at all hours of the day or evening, old Tim could be seen sitting before the cabin, his arms around the boy…. And sometimes, holding the banjo steady, he would invite little Tim to try his tiny hands at picking the strings" (*8*).

Tanner's empathetic brush captures the intimate moment. Head lowered attentively, the old man surrounds the child, who is at ease and receptive. On the floor lie the implements of their modest life. Faithful to the story, a "long panel of light" bares the cabin's "smoke-stained wall." The artist's masterful technique is rivaled only by the dignity of the scene: gently handing down a prized possession, a music lesson, a life lesson.

In genre as in biblical scenes, Tanner manipulated light to create emotion and drama. In The Banjo Lesson he lights up the interior of a run-down dwelling to reveal what might otherwise be missed: the poor, glowing with humanity and knowledge. These qualities, so clearly expressed in art, can also be seen in the light of science. But because they defy mathematical measurement, they become invisible in cost-effectiveness and other studies, eluding economic analysis and adequate attention.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

High Rate of Mobilization for bla_{CTX-M}s

Mycobacterium ulcerans Disease in Peru

Chikungunya Fever in Travelers Returning from Indian Ocean Region, 2006

Rescinding Community Mitigation Strategies in an Influenza Pandemic

Nontuberculous Mycobacterial Infections among Cystic Fibrosis Patients, Israel

Mycobacterium xenopi Determinants, the Netherlands

Increased Mortality Rate Associated with Chikungunya Epidemic, Ahmedabad, India

Genetic Variability of West Nile Virus in US Blood Donors, 2002–2005

Molecular Epidemiology of Eastern Equine Encephalitis Virus, New York

Evidence of Exposure to Novel Parainfluenza Virus in 2 Bottlenose Dolphin Populations

Integrated Food Chain Surveillance System for *Salmonella* spp., Mexico

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Protective Effect of Maritime Quarantine in South Pacific Jurisdictions, 1918–19 Influenza Pandemic

Mutations in Influenza A Virus (H5N1) and Possible Limited Spread, Turkey, 2006

Dolphin Morbillivirus Epizootic Resurgence in the Mediterranean

Screening Pneumonia Patients for Mimivirus

Hemagglutinating Encephalomyelitis Coronavirus Infection in Pigs, Argentina

Staphylococcus aureus of Sequence Type 398 in Pigs and Humans

Complete list of articles in the March issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 3–6, 2008
15th Conference on Retroviruses and Opportunistic Infections
Hynes Convention Center
Boston, MA, USA
http://www.retroconference.org

March 10–13, 2008
2008 National STD Prevention Conference: Confronting Challenges, Applying Solutions
Hilton Chicago
Chicago, IL
http://www.cdc.gov/stdconference/

March 16–19, 2008
International Conference on Emerging Infectious Diseases
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

March 28–30, 2008
Clinical Infectious Disease Update Course 2008–Eleventh Annual Management Review for the Practicing Physician
The Grand Hyatt
New York, NY, USA
http://www.cbcbiomed.com

April 5–8, 2008
Society for Healthcare Epidemiology of America (SHEA) 18th Annual Scientific Meeting
Buena Vista Palace
Orlando, FL, USA
Abstract submission deadline: January 4, 2008
http://www.shea-online.org

April 8–11, 2008
Genomes 2008 - Functional Genomics of Microorganisms
Institut Pasteur
Paris, France
http://www.pasteur.fr/infosci/conf/sb/genomes_2008

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Announcements may be posted on the journal Web page only, depending on the event date.

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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January 2008

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

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Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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