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Moschophoros (Calf-Bearer)
attributed to Phaidimos.
Statue of the patriot Romvos offering sacrificial calf to Athena.
c. 570 BCE. Marble. Height 165 cm.
No. 624 Acropolis Museum,
Athens, Greece

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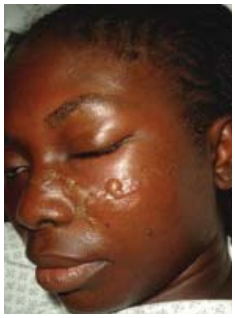

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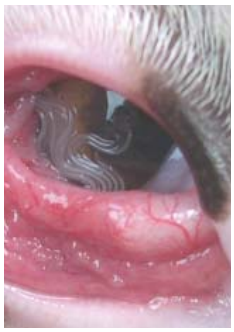

Letters

Book Reviews

News & Notes

Bluetongue Epidemiology in the European Union

Claude Saegerman,* Dirk Berkvens,† and Philip S. Mellor‡

Bluetongue (BT) is a reportable disease of considerable socioeconomic concern and of major importance in the international trade of animals and animal products. Before 1998, BT was considered an exotic disease in Europe. From 1998 through 2005, at least 6 BT virus strains belonging to 5 serotypes (BTV-1, BTV-2, BTV-4, BTV-9, and BTV-16) were continuously present in the Mediterranean Basin. Since August 2006, BTV-8 has caused a severe epizootic of BT in northern Europe. The widespread recrudescence and extension of BTV-8 infections in northern Europe during 2007 suggest that requirements for BTV establishment may now be fulfilled in this area. In addition, the radial extension of BTV-8 across Europe increases the risk for an encounter between this serotype and others, particularly those that occur in the Mediterranean Basin, where vector activity continues for more of the year. This condition increases the risk for reassortment of individual BTV gene segments.

Bluetongue (BT) is an infectious but noncontagious viral disease caused by *Bluetongue virus* (BTV). The virus belongs to the family *Reoviridae*, genus *Orbivirus*; there are 24 serotypes (*1*). The viral genome consists of 10 double-stranded RNA segments that encode for 4 nonstructural proteins (NS1, NS2, NS3, and NS3A) and 7 structural (VP1-VP7) proteins (*2*,*3*). BTV serotypes 1, 2, 3, 4, 6, and 10 have a high pathogenic index and high epidemic potential (*4*). However, a high genetic diversity of BTV exists that is a consequence of both drift (i.e., point mutations) and shift (i.e., reassortment of individual BTV gene segments) so pathogenicity even within a serotype may be highly variable (*5*).

BT is a World Organization for Animal Health reportable disease and is of considerable socioeconomic concern and of major importance in the international trade of animals and animal products (*4*). Before 1998, BT was considered an exotic disease in Europe with just a few sporadic incursions (e.g., Spain and Portugal from 1956 through 1960) (*6*).

Our aim in this article is to provide a synthesis and some perspectives of BT epidemiology in the European Union (EU) since BTV's introduction in 1998. To this effect, we provide a short overview of the epidemiologic situation in Europe, followed by a brief description of the susceptible species, a discussion of the vectorial capacity and competence of the *Culicoides* spp. vectors, and an outline of the modes of introduction and mechanisms of amplification.

Epidemiologic Situation in Europe

BTV in EU, 1998–2005

During this 8-year period, at least 6 BTV strains belonging to 5 serotypes (BTV-1, BTV-2, BTV-4, BTV-9, and BTV-16) have been continuously present in parts of the Mediterranean Basin, including several member states of the EU (Table, Figure 1) (*1*,*5*, *7*–*12*). This emergence of BT into parts of Europe never before affected was attributed mainly to climate change and was linked to the northern expansion of the major Old World vector *Culicoides imicola* (Kieffer), which is an Afro-Asiatic species of biting midge (*13*). Additionally, novel indigenous European vector species of *Culicoides* within the *Obsoletus* and *Pulicaris* complexes were involved.

In the Mediterranean Basin 2 epidemiologic systems seem to predominate. The first one is located in the eastern part of the basin, where serotypes 1, 4, 9, and 16 were identified. In this system, the BTV strains originated in the Near, Middle, or Far East. The vectors included other species of *Culicoides* in addition to *C. imicola*. This finding was deduced from the fact that the disease penetrated into areas where *C. imicola* does not occur (the Balkans and beyond) (*9*). The involvement of novel vectors was subsequently confirmed when the causative virus was isolated from mixed pools of 2 species, *C. obsoletus* (Meigen) and

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Table. Outbreaks of bluetongue in Europe, 1998–2005*†

Country	Year of first outbreak	BTV serotype(s)	Main suspected or identified vector(s)
Albania	2002	9	*Culicoides obsoletus, C. pulicaris*
Bosnia–Herzegovina	2002	9	ND
Bulgaria	1999	9	*C. obsoletus, C. pulicaris*
Croatia	2001	9, 16	*C. obsoletus, C. scoticus*
Cyprus	2003	16	*C. imicola, C. obsoletus,*
Former Yugoslav Republic of Macedonia	2001	9	ND
France (Corsica)	2000	2, 4, 16‡	*C. imicola, C. pulicaris, C. obsoletus*
Greece	1998	1, 4, 9, 16	*C. imicola, C. obsoletus*
Italy	2000	1, 2, 4, 9, 16	*C. imicola, C. obsoletus, C. pulicaris*
Kosovo	2001	9	ND
Montenegro	2001	9	ND
Portugal	2004	2,§ 4	*C. imicola, C. obsoletus, C. pulicaris*
Serbia	2001	9	ND
Spain	2000	2	*C. imicola,C. obsoletus, C. pulicaris*
Turkey	1998	4, 9, 16	*C. imicola,C. obsoletus, C. pulicaris*

*BTV, Bluetongue virus; ND, no data recorded.
†Sources: (*5,7–10*).
‡This is an insufficiently attenuated vaccine strain (*11*).
§This strain is indistinguishable from Onderstepoort BTV-2 live attenuated vaccine strain (*7*).

C. scoticus (Downes and Kettle), collected in central Italy (*14*) and from *C. pulicaris* (Linnaeus) in Sicily (*15*). The second epidemiologic system comprises the western part of the Mediterranean Basin, where serotypes BTV-1, BTV-2, BTV-4, and BTV-16 were identified and the main vector is *C. imicola*. Although the appearance of BTV serotype 16 in this *C. imicola* system is the result of the westward spread of the virus across Europe (*16*), it is of particular interest because of strong indications that the field virus may represent a reversion to virulence of the attenuated vaccine (e.g., in Corsica and in Sardinia in 2004, strains of BTV-16 isolated from the field were identical to the live attenuated monovalent vaccine strain) (*1*,*11*) (Table, Figure 1).

BT in Central and Northern Europe, mid-August 2006 to late December 2007

BT was first identified in northern Europe in August 2006 and can be defined as an emergent disease in this zone (*17*). Between the date of the first report (August 17, 2006) and February 1, 2007 (*18*), 2,122 BT cases were entered into the European Commission's Animal Disease Notification System (ADNS) (http://ec.europa.eu/food/animal/diseases/adns/index_en.htm) (Figure 2) (*19*). In this region, in 2006, a pool of 50 nonengorged, parous *C. dewulfi* (Goetghebuer) in the Netherlands were positive by PCR for BTV (*20*), and several pools of *C. obsoletus* complex in Germany (i.e., not identified down to species) were also PCR positive for BTV (*21*) (Figure 3). Although isolation of live BTV was not attempted in either instance, this research, conducted in an area where *C. imicola* does not occur, confirms the earlier findings of Mellor and Pitzolis, who isolated infectious BTV from nonengorged parous *C. obsoletus* in Cyprus, and shows that indigenous European *Culicoides* species can support a BT epizootic (*22*). Because *C. obsoletus* complex midges and *C. dewulfi* occur widely across central and northern Europe, this entire area must now be considered to be at risk for BTV (*23*,*24*).

Moreover, in relation to the demonstrated overwintering ability of the virus in northern Europe, small numbers of adult *Culicoides* spp. were captured in animal housing during the winter period (November 25, 2006, to March 9, 2007) (i.e., females of *C. obsoletus complex*, males of *C. obsoletus*, *C. scoticus*, and *C. dewulfi*) (*25*). Whether the occurrence of these midges and the possibility of their activity extending over the winter in such climatically protected locations can explain the persistence of virus from 1 vector transmission season to the next (*13*) or whether they represent newly emerged midges from nearby breeding sites is not known (*25*). Several hypotheses have been formulated to explain the overwintering ability of BTV: by persistence

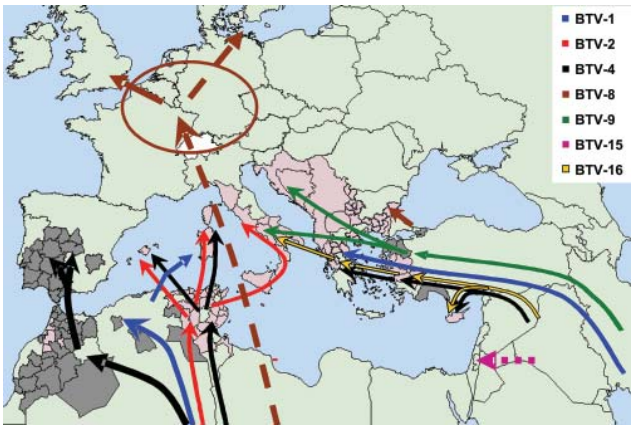

Figure 1. The molecular epidemiology of bluetongue virus (BTV) since 1998: routes of introduction of different serotypes and individual virus strains. *Presence of BTV-specific neutralizing antibodies in animals in Bulgaria, but the presence of BTV serotype 8 cannot yet be confirmed.

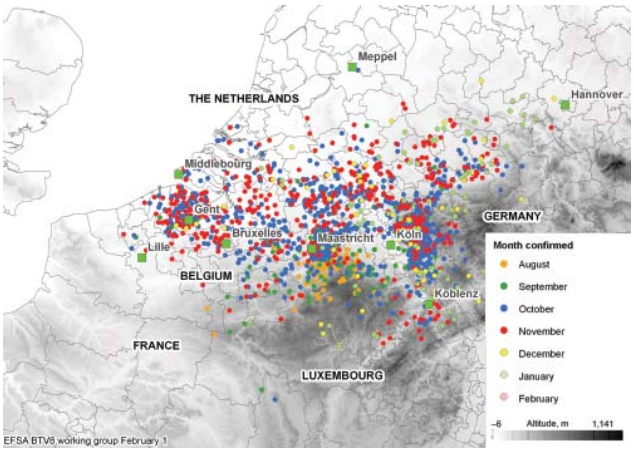

Figure 2. Monthly distribution of confirmed bluetongue virus 8 (BTV-8) outbreaks in northern and central Europe from August 17, 2006, through February 1, 2007. After January 1, 2007, few BTV cases were reported; those that were probably involved animals that had been infected, but not detected, in 2006.

within surviving adult vectors themselves, transovarial transmission through the vector, or prolonged/persistent infection in viremic or aviremic vertebrate hosts (*13*,*25*,*26*).

The focus of interest now is to see if BTV is able to survive regularly between vector seasons and become endemic to northern Europe. The recrudescence of BTV-8 in northern France, the Netherlands, Belgium, Luxembourg, and Germany in 2007, and also the emergence of BTV-8 in the United Kingdom, Denmark, Switzerland, and the Czech Republic, suggests that this may well be the case (*27*,*28*). Unlike farther south, where populations of the traditional vector, *C. imicola,* peak in the late summer and autumn, when most BT cases occur, populations of the indigenous European vectors peak earlier in the year; whether this will be reflected in a change in the temporal occurrence of BT cases remains to be seen. In the period from January 1, 2006, through December 28, 2007, 12 EU member states and Switzerland reported BT outbreaks on their territories, comprising all of the serotypes reported in Europe since 1998 (Figure 4) (*29*,*30*).

Susceptible Species

BTV is transmitted between its ruminant hosts almost exclusively through the bites of the females of vector species of the *Culicoides* biting midge (*31*). The global distribution of BTV, therefore, is restricted to those regions where these vector species of *Culicoides* occur, and its transmission period is limited to the times when adult vectors are active. Depending on the species, adult vector activity generally starts some time in spring. Activity is positively correlated with temperature and reaches a maximum between 28°C and 30°C; activity decreases when the temperature drops and, for the traditional Afro-Asiatic vector *C. imicola*, is probably nonexistent at temperatures <10°C (*13*,*31*).

BTV can infect a broad spectrum of domestic and wild ruminants. However, serious clinical signs have been observed only in certain breeds of sheep (improved breeds) and a few deer species (*32*,*33*). Cattle and goats usually exhibit subclinical infections and therefore may serve as important and covert viral reservoirs for sheep (*32*). However, some serotypes such as serotype 8, which recently caused infection in northern Europe, exhibit a more important virulence in cattle (*34*,*35*) with serious socioeconomic consequences (*5*).

Vector Capacity and Competence

Risk for BTV infection is linked closely to the presence of adult vector *Culicoides* spp. (*31*). Until recently, *C. imicola* was believed to be the only important vector of BTV in southern Europe, but it is now known that several, newly recognized vector species are also involved. Others may be identified in the future.

Vector competence of an insect species and vector capacity of an insect population are important parameters in this respect (*36*). Vector competence is the (innate) ability of a vector to acquire a pathogen, maintain it, and successfully transmit it to a susceptible host (*13*). Vector competence may be determined in the laboratory by providing groups of insects of a particular species with blood meals of appropriate concentrations of virus and assessing infection and transmission rates. Vector competence is defined as the proportion of feeding insects that support virus repli-

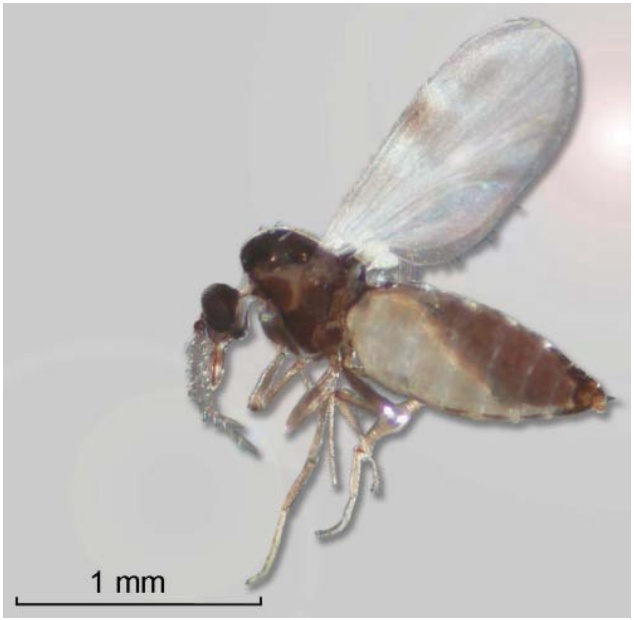

Figure 3. A gravid female *Culicoides dewulfi* collected from a location near bluetongue outbreaks in Belgium in 2006 (Photograph: Reginald De Deken and Maxime Madder, Institute of Tropical Medicine, Antwerp, Belgium).

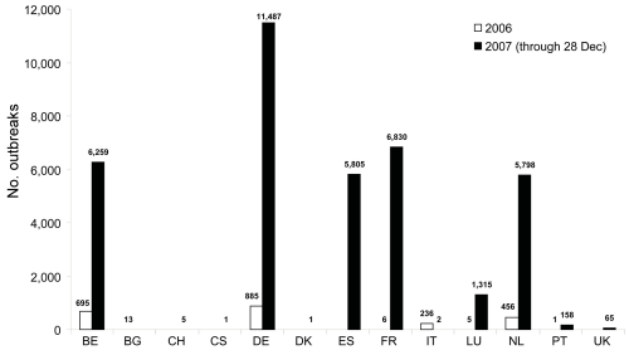

Figure 4. Number of bluetongue outbreaks in Europe since January 1, 2006 (all serotypes). BE, Belgium; BG, Bulgaria; CH, Switzerland; CS, Czech Republic; DE, Germany; DK, Denmark; ES, Spain; FR, France; IT, Italy; LU, Luxembourg; NL, Netherlands; PT, Portugal; UK, United Kingdom.

cation and transmit virus after a suitable incubation period. In situations where transmission is difficult to demonstrate because of the technical problems in refeeding "difficult" insects such as *Culicoides* spp., it has become established practice to assume transmission if virus can be recovered from the salivary glands.

Vector capacity refers to the potential for virus transmission of an insect population and takes into account a range of insect, host, and environmental variables, including vector abundance, vector survival, biting and transmission rates, host preferences, and host abundances, under a range of external (e.g., bioclimatic) conditions. Vector capacity can be defined as the number of infective bites that an infected vector causes during its lifetime (usually 2–4 weeks in the case of vector species *Culicoides*) (*36*,*37*).

Determining the 2 parameters explained above is essential to accurately estimate vector transmission rates and predict whether BTV will become established in an area. Such detailed studies inevitably demand substantial financial and scientific resources and require a multidisciplinary approach.

Modes of Introduction and Mechanisms of Amplification

Introduction of BTV from 1 area into another can occur in 4 ways: through animal movement (domestic and wild ruminants) or animal product transport (semen, embryos); by infected vector *Culicoides* spp. carried by various living (plants, animals) or inanimate (airplanes, ships) means; through the active flight of infected vector *Culicoides* spp. (local propagation); and through passive flight of infected vector *Culicoides* spp. on the wind (responsible for long-distance dissemination).

Whether the virus becomes established in a new area depends upon the number and distribution of susceptible hosts, the duration and titer of the BTV viremia in the hosts, the vector capacity of the local vector population, and the ambient temperature. In essence, establishment depends upon a sufficient number of vector *Culicoides* spp. becoming infected by feeding upon local viremic hosts, surviving long enough to ensure completion of the intrinsic incubation period (4–20 days, depending on ambient temperature), and transmitting the virus by bite to new hosts (*13*). The extrinsic incubation period is the interval between when a vector is infected and when it first becomes capable of transmitting the BTV to a new host (*38*). These requirements for BTV establishment have clearly been fulfilled in much of southern Europe, as BTV has survived there in many locations since the late 1990s.

Conclusions

The widespread recrudescence of BTV-8 infections in northern France, Belgium, the Netherlands, Luxembourg, and Germany in 2007 and the emergence of BTV-8 in the United Kingdom, Denmark, Switzerland, and the Czech Republic in the same year suggest that the requirements for BTV establishment may now also be fulfilled in many more

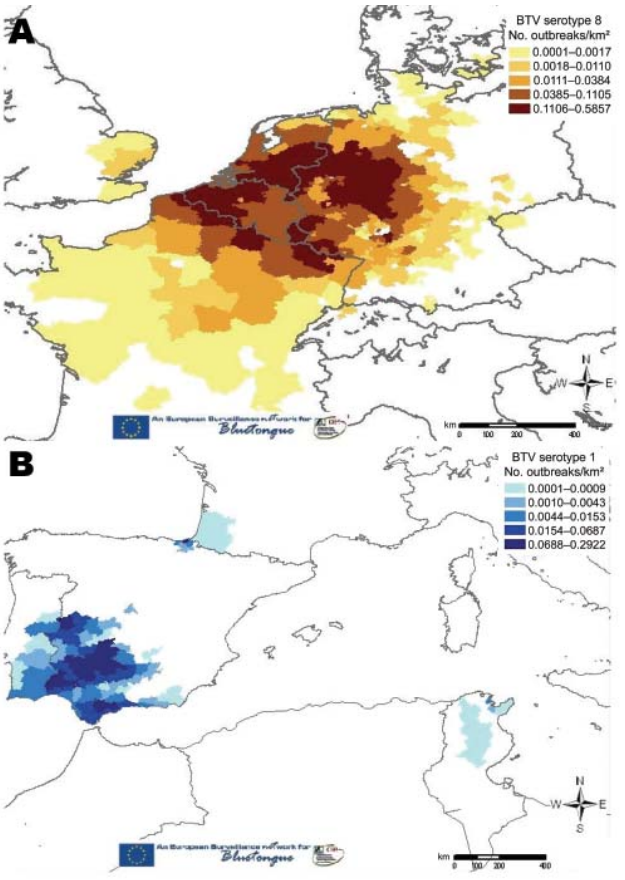

Figure 5. Number of bluetongue virus (BTV) outbreaks caused by BTV-8 (A) and BTV-1 (B) per kilometer (quartile scale) from May 1, 2007, to December 28, 2007 (EU-BTNET system; available from http://eubtnet.izs.it/btnet).

northerly and central parts of Europe (in the absence of *C. imicola*). In addition, the radial extension of BTV-8 across Europe (including the jump across the English Channel) (Figure 5) (*39*) increases the risk for an encounter between this serotype and others, particularly those that occur in the Mediterranean Basin (second epidemiologic system). BTV serotypes 1, 2, 4, and 16 have been identified in this area, and the addition of a further serotype will considerably increase the potential for reassortment between these viruses (Figure 6) (*27*,*40*). Indeed, the number of possible reassortments in the case of BTV, which has 10 segments, increases with the number of cocirculating serotypes (e.g., 1,024 for 2 serotypes [2^{10}] and 59,049 for 3 serotypes [3^{10}]) (*4*). Moreover, the phenomenon of reassortment has already been demonstrated during the 1998–2005 BTV outbreaks in Europe (*5*).

Furthermore, in the southern epidemiologic system, *C. imicola,* the Afro-Asiatic vector of BTV, occurs in addition to the *C. obsoletus* complex. As the population abundance of *C. imicola* peaks later in the year than the *Obsoletus* complex, this means that virus may be transmitted for a much greater portion of the year.

With regard to prophylaxis, possibly the best strategic option for control of clinical BT outbreaks in the European endemic areas is vaccination of susceptible animals with inactivated vaccines to protect against disease and to exclude the possibility of reversion to virulence of the vaccine

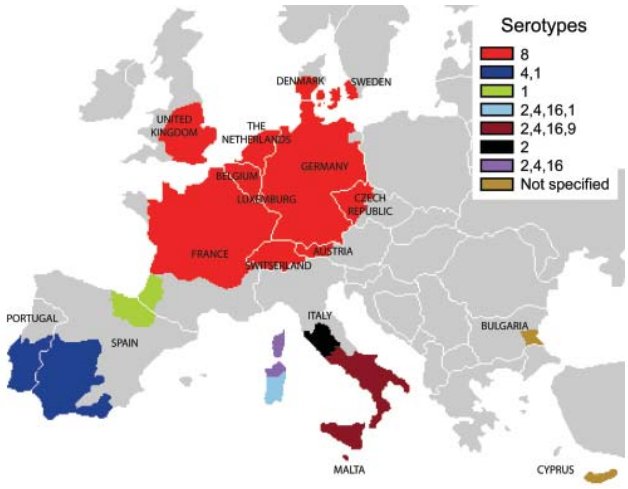

Figure 6. Bluetongue virus (BTV) restriction zones in Europe, by serotype. The radial extension of BTV-8 across Europe increases the risk for an encounter between this serotype and other serotypes that occur in the Mediterranean Basin (second epidemiologic system, where serotypes BTV-1, BTV-2, BTV-4, and BTV-16 were identified and the main vector is *Culicoides imicola*). This situation increases the risk for reassortment of individual BTV gene segments, and, in the more southerly areas, the period of vector activity is also likely to extend, leading to a longer BTV-8 season. In addition, BTV-1, which was first identified in sheep with clinical signs of BT in the south of the Iberian Peninsula in July 2007, has extended its range into northern Spain and southwestern France (Pyrénées-Atlantiques), since November 2007; this ongoing expansion is matter of major concern.

viruses and reassortment between vaccine and field strains of the virus (*4*,*5*). Veterinary authorities and legislators throughout northern Europe would do well take note of these recent and considerable changes in the epidemiology of BT.

Acknowledgments

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Dr Saegerman is professor of epidemiology and risk analysis applied to veterinary sciences in the Faculty of Veterinary Medicine, University of Liege. His interests include field epidemiology studies of animal diseases and risk evaluation in the food chain, including primary production.

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Potential Use of Antiviral Agents in Polio Eradication

Armando M. De Palma,* Gerhard Pürstinger,† Eva Wimmer,† Amy K. Patick,‡ Koen Andries,§ Bart Rombaut,¶ Erik De Clercq,* and Johan Neyts*

In 1988, the World Health Assembly launched the Global Polio Eradication Initiative, which aimed to use large-scale vaccination with the oral vaccine to eradicate polio worldwide by the year 2000. Although important progress has been made, polio remains endemic in several countries. Also, the current control measures will likely be inadequate to deal with problems that may arise in the postpolio era. A panel convoked by the National Research Council concluded that the use of antiviral drugs may be essential in the polio eradication strategy. We here report on a comparative study of the antipoliovirus activity of a selection of molecules that have previously been reported to be inhibitors of picornavirus replication and discuss their potential use, alone or in combination, for the treatment or prophylaxis of poliovirus infection.

The Global Polio Eradication Initiative (GPEI) was launched by the World Health Assembly 20 years ago. The principal idea behind the GPEI was to eliminate polio worldwide by the year 2000 by means of large-scale vaccination with the oral live attenuated polio vaccine (OPV) developed by Albert Sabin (*1*). The GPEI has resulted, since 1988, in a decrease in poliomyelitis cases from 350,000 to <2,000 (*2*,*3*). Today, poliovirus (PV) is endemic in 4 countries (Nigeria, India, Pakistan, and Afghanistan), whereas the virus was prevalent in >125 countries at the time the initiative was launched (*4*). When wild PV transmission has been interrupted, the World Health Organization proposes ending the global routine OPV to prevent the risk for vaccine-associated paralytic poliomyelitis, chronic infection of immunodeficient persons, and the reestablishment of poliomyelitis through circulating vaccine-derived PV (*5*). A panel was convened by the National Research Council to evaluate the potential for an antiviral drug as one of the tools to minimize poliomyelitis risk after OPV cessation. The conclusion of the panel was that it would be appropriate, and possibly essential, to develop antiviral drugs for PV infection, as an additional tool to address the problems that might arise in the "post-polio" era (*6*). Antiviral agents do not confer immunity but could be used prophylactically as well as therapeutically. They could protect inactivated polio vaccine (IPV) recipients from PV infection, limit spread until immunity can be ensured and help clear vaccine-derived PV from persistently infected persons (*7*). The ideal drug would be safe, inexpensive, easy to use, stable, and manifest broad activity toward PV strains.

To date, few, if any, drug discovery programs for PV have been initiated. Therefore, research initiatives leading to the successful development of anti-PV drugs will have to rely on the current knowledge of existing picornavirus antiviral agents. Antipicornavirus compounds that reached clinical trials are scarce, and despite the fact that some of these drugs have demonstrated activity against certain picornavirus-associated conditions in humans, no specific antipicornavirus agent has yet been approved by the US Food and Drug Administration (FDA) (*8*).

A substantial number of small molecule compounds have been reported as potent inhibitors of the replication of picornaviruses in vitro (*8*). These compounds could serve as scaffolds for the development of more potent and selective inhibitors of PV. The information available on their structure-activity relationship and their mechanism of action could be exploited as a solid base for developing a specific anti-PV therapy.

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We report on a comparative study of a selected series of antipicornavirus drugs for their ability to inhibit PV replication in vitro. The unique aspect of this report lies in the fact that 1) certain drugs (e.g., rupintrivir) were specifically developed to treat rhinovirus and other infections and have never been evaluated for their ability to block PV replication and 2) the selected compounds have never been compared in parallel by using the same technique against the 3 vaccine strains.

Rationale for Selection of Antipicornavirus Drugs

Because this study was triggered by the recognition that antiviral drugs will be needed in the postvaccination era as a countermeasure against the persistence or reemergence in the environment of vaccine-associated virus, we decided to confine our study to the 3 Sabin strains used for vaccination. The aim was to include compounds that act on different targets in the picornavirus replication cycle (preferably 1 or 2 compounds per target) (Figure 1). When a rather large number of molecules had been described that act through the same target (e.g., for the capsid binding agents), we selected those compounds that were in the most advanced state of development and preferably had been studied in a clinical setting. When only 1 or a few compounds had been described for a particular target (for example, with enviroxime, the sole protein 3A–targeting drug reported so far), the impact in the clinical setting was considered less important. Ribavirin was included as a reference standard, since it was regarded as a broad-spectrum inhibitor of positive-strand RNA viruses.

Methods

The antiviral and cytotoxic activities of the selected compounds were initially determined by means of a cell protection assay. In this assay, a soluble tetrazolium compound (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium [MTS]), when used in combination with an electron transfer reagent (phenazine methosulfate [PMS]) is bioreduced by viable cells in culture, a reaction that is colorimetrically quantified. The antiviral and cytotoxic activities were expressed as mean effective concentration (EC_{50}, the compound concentration that inhibits virus-induced cytopathic effect [CPE] formation by 50%) and 50% cytotoxic concentration (CC_{50}). Therapeutic indexes (TIs) were expressed as the ratio between the CC_{50} and the EC_{50}.

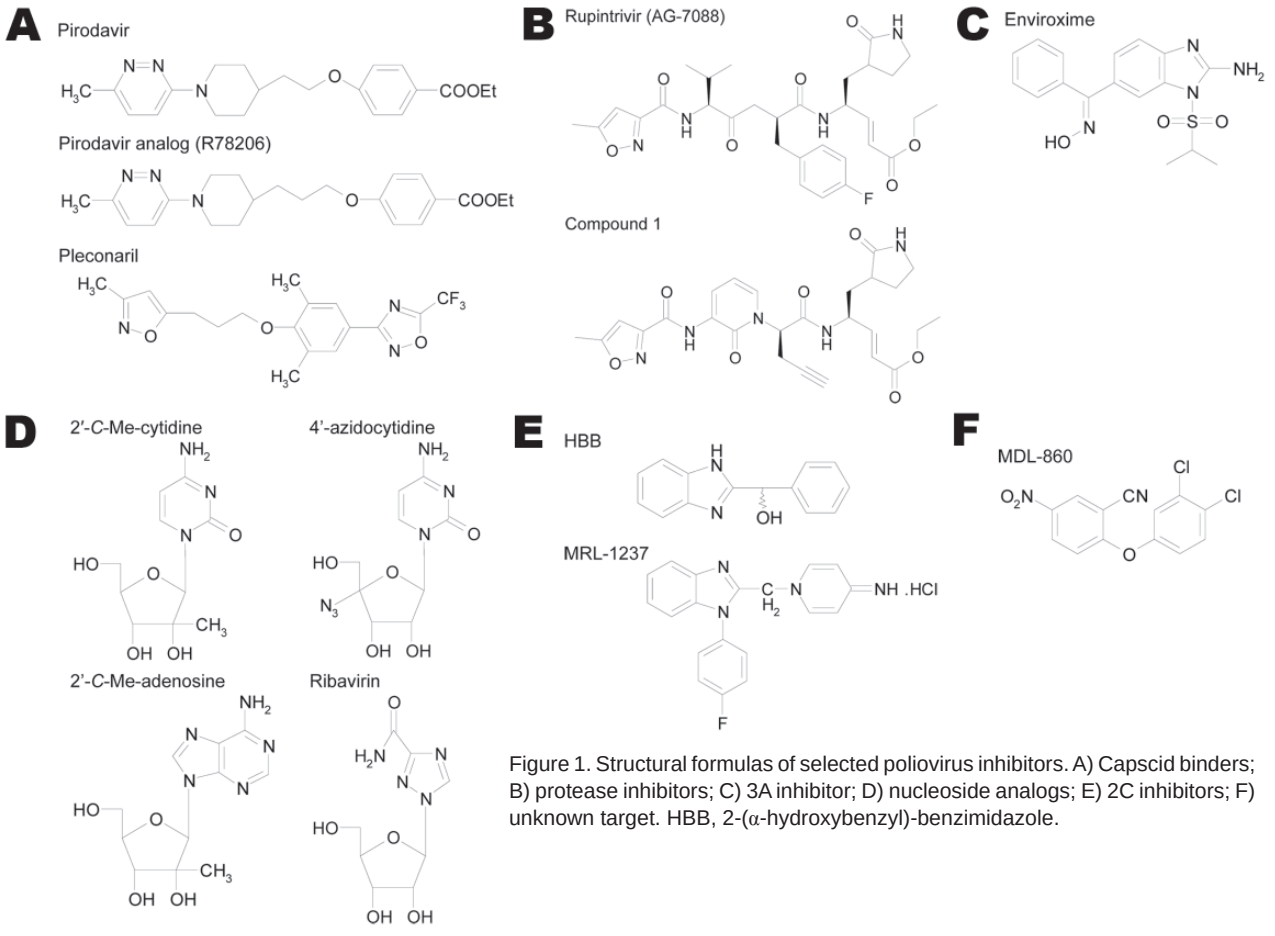

Figure 1. Structural formulas of selected poliovirus inhibitors. A) Capscid binders; B) protease inhibitors; C) 3A inhibitor; D) nucleoside analogs; E) 2C inhibitors; F) unknown target. HBB, 2-(α-hydroxybenzyl)-benzimidazole.

Briefly, HeLa cells (ATCC CCL-2), grown to confluency in 96-well plates, were infected with 100 50% cell culture infectious doses of virus. After an adsorption period of 2 hours at 37°C, unadsorbed virus was removed and serial dilutions of the compounds were added. The cultures were further incubated at 37°C for 3 days, until complete CPE was observed in the infected and untreated virus control. For cytotoxicity determination, uninfected cultures were incubated with dilution series of compound for 3 days at 37°C. After removal of the medium, 90 μL medium and 10 μL MTS/PMS (Promega, Leiden, the Netherlands) were added to each well; after an incubation period of 2 hours at 37°C, the optical density of each well was read at 498 nm in a microplate reader. EC_{50} values were calculated as previously described (*9*).

Results

Capsid-binding Agents

One of the best studied targets for antiviral therapy in picornaviruses is a hydrophobic pocket underneath the canyon floor that surrounds each 5-fold axis of the viral capsid. Binding of specific inhibitors into this pocket increases virion rigidity, thus inhibiting attachment or disassembly of the viral particle after receptor binding (*10*). Consequently, the release of the viral genome into the host cell is prevented and viral replication is inhibited. Two of the most extensively characterized series of capsid-binding agents are the so-called WIN compounds, developed by Sterling Winthrop (New York, NY, USA), and a series of pyridazine analogs developed by the Janssen Research Foundation (Beerse, Belgium). The prototypes of these series are pleconaril (*11*) and pirodavir (*12*), respectively.

In clinical studies, pleconaril was a promising candidate for treating the common cold, but it was disapproved by the FDA in 2002, mainly because of possible interactions with other drugs, including those for birth control. Soon thereafter, pleconaril was licensed to Schering-Plough, which in 2007 completed a phase II clinical trial to study the effects of pleconaril nasal spray on common cold symptoms and asthma exacerbations following human rhinovirus (HRV) exposure. Meanwhile, pleconaril is still being used successfully on a compassionate basis for treating life-threatening enterovirus infections in children (*13*). Notably, it was effective in stopping virus excretion in a child persistently infected with PV, when combined with gamma globulin–mediated virus clearance (*14*). In another trial with a persistently infected person, however, treatment produced no benefit (*7*).

Intranasal pirodavir (R77975) was active in some clinical trials of human experimental rhinovirus infections, but lack of therapeutic efficacy and metabolic instability after oral administration halted further development. As shown in the Table, pleconaril and pirodavir, as well as a pirodavir analog (R78206) (*15*) inhibited PV2 and PV3 replication with EC_{50} values <2 μmol/L and TIs of 60 to >179. However, only R78206 exhibited inhibitory activity against PV1. Pirodavir proved 5- to 20-fold less active on PV1 replication, and pleconaril was inactive up to the highest concentration tested.

Table. Inhibitory activity of selected compounds against replication of poliovirus Sabin strains 1, 2, and 3 in HeLa cells, as determined by a CPE reduction assay*

Compound	EC_{50} (μmol/L)			Toxicity (CC_{50};μmol/L)	TI (min–max)
	PV1	PV2	PV3		
Capsid binders					
Pirodavir analog (R78206)	0.76 ± 0.18	0.22 ± 0.19	0.11 ± 0.10	27 ± 34	35–245
Pleconaril	>100	1.1 ± 0.6	0.22 ± 0.15	66 ± 6	<0.66–300
Pirodavir (R77975)	10 ± 1	1.7 ± 0.1	0.56 ± 0.03	>100	>10–>179
Protease inhibitors					
Rupintrivir	0.022 ± 0.028	0.041 ± 0.024	0.0052 ± 0.0046	>100	>2,439–>19,231
Compound 1	0.26 ± 0.24	0.31 ± 0 .21	0.060 ± 0 .000	>100	>322–>1,667
3A inhibitor					
Enviroxime	0.2 ± 0.25	0.056 ± 0.020	0.035 ± 0.029	58 ± 6	290–1,657
Nucleoside analogs					
Ribavirin	57 ± 13	64 ± 4	55 ± 7	>100	>1.6–>1.8
2'-*C*-methylcytidine†	15 ± 18	29 ± 27	3.9 ± 2.3	>100	>3.4–>26
2'-*C*-methyladenosine	5.5 ± 0.0	5.6 ± 0.1	5.4 ± 0.4	84 ± 0	15
4'-azidocytidine	>100	>100	>100	>100	><1
2C inhibitors					
HBB	300 ± 68	225 ± 128	295 ± 88	>400	>1.3–>1.8
MRL-1237	5.3 ± 0.3	4.6 ± 1.4	3.8 ± 2.5	>100	>19–>26
Unknown target					
MDL-860	6.0 ± 1.6	3.6 ± 2.2	2.2 ± 1.5	>100	>17–>45

*CPE, cytopathic effect; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; PV, poliovirus; TI, therapeutic index (CC_{50}/EC_{50}); min, minimum; max, maximum; HBB, 2-(α-hydroxybenzyl)-benzimidazole.
†Valopicitabine (oral valine ester prodrug of 2′-*C*-methylcytidine).

Protease Inhibitors

A second approach to inhibiting PV replication is by targeting the virus-encoded proteases 2A and/or 3C. These enzymes cleave the single polyprotein, encoded by the PV genome, into mature proteins. Rupintrivir (AG-7088, Pfizer, New York, NY, USA) is an irreversible inhibitor of the 3C function (*16*,*17*). Despite some successful trials in patients that were experimentally infected with HRV, rupintrivir was not able to mitigate disease severity in studies of natural rhinovirus infection, and clinical development was stopped (*18*). Further efforts by Pfizer resulted in the development of compound 1, an inhibitor with a similar mechanism of action and with an excellent oral bioavailability (*18*). Both compounds inhibited all 3 PV strains with EC_{50}s <1 μmol/L and TIs of >322 to >19,230 (Table). Rupintrivir was the most potent compound of the selected series with EC_{50} values in the nanomolar range (5–40 nmol/L) against each of the 3 tested PV strains (Table).

Protein 3A Inhibitors

Enviroxime is a benzimidazole derivative that inhibits the replication of enteroviruses and rhinoviruses in vitro by targeting the nonstructural protein 3A (*19*,*20*). Enviroxime inhibited the replication of all 3 strains of PV, with EC_{50} values of 35–200 nmol/L and TIs of 290–1,657 (Table). Previous in vivo studies with enviroxime, however, have shown toxicity and only weak to moderate activity, due to poor solubility and pharmacokinetics (*21*–*24*). Structural derivatives of enviroxime such as the C_2- and vinylacetylene analogs were reported to have a better oral bioavailability and pharmacologic profile (*25*,*26*) and may therefore be considered as leading candidates for further development.

Nucleoside Analogs

The nucleoside analog ribavirin is an antiviral drug with broad-spectrum activity against RNA and DNA viruses. Ribavirin is used in combination with interferon in the treatment of hepatitis C virus (HCV) infection (*27*) and as an aerosol to treat respiratory syncytial virus infections in children (*28*). As expected, ribavirin proved to be a weak inhibitor of PV replication with EC_{50} values of 50–60 μmol/L (TIs >1.6). Valopicitabine is the oral valine ester prodrug of another nucleoside analog, 2′-*C*-methylcytidine. The 5′-triphosphate of 2′-*C*-methylcytidine is an inhibitor of HCV polymerase (*29*). Clinical development of valopicitabine for the treatment of HCV infection was recently stopped, mainly because of gastrointestinal side effects. The compound was shown to exhibit relatively broad-spectrum activity against positive-sense single-stranded RNA viruses, including inhibition of the replication of foot-and-mouth-disease virus (*30*). It can be assumed that the mechanism by which 2′-*C*-methylcytidine inhibits picornaviruses is also by inhibition of the viral polymerase. As shown in the Table, 2′-*C*-methylcytidine inhibited the replication of PV strains with EC_{50} values of 3.9–29 μmol/L (TIs >3.4–>25.6). The adenosine analog of valopicitabine, as well as another nucleoside analog, 4′-azidocytidine (a potent inhibitor of HCV replication) were also included in this study. 2′-*C*-methyladenosine proved equipotent (≈5 μmol/L) against all 3 PV strains; whereas 4′-azidocytidine proved inactive (Table).

Protein 2C Inhibitors

MRL-1237 and 2-(α-hydroxybenzyl)-benzimidazole (HBB) are inhibitors that target the enteroviral nonstructural protein 2C (*31*,*32*). MRL-1237 showed antiviral activity against PV strains 1, 2, and 3 with TIs >19. HBB appeared to be a weak inhibitor of PV replication with EC_{50}s of 200–300 μmol/L and TIs >1.3.

Compounds with Unknown Mechanism of Action

Compound MDL-860 was discovered as a broad-spectrum inhibitor of picornavirus replication, although the precise mechanism of antiviral activity has never been unraveled (*33*). The anti-PV activity of MDL-860 proved comparable to that of the 2C inhibitor MRL-1237.

To further confirm the activity observed in the CPE reduction assays, infectious virus yield reduction assays were carried out on the supernatant of PV1-infected cultures. As depicted in Figure 2, rupintrivir, the most active compound in the CPE reduction assay, caused a 6-log_{10} decrease of infectious virus production at 100 μmol/L, and reduced virus progeny formation 10–1,000-fold at concentrations of 10–100 nmol/L. Conversely, pleconaril, which did not protect against PV1-induced CPE formation, was not able to reduce infectious virus yield. A similar correlation between CPE formation and infectious virus production was observed for all other compounds included in the study (data not shown).

Discussion and Perspectives

From our comparative study, rupintrivir and its analog compound 1 emerged as highly potent and broad-spectrum anti-PV compounds, without any signs of cytotoxicity up to the highest concentrations tested. The in vitro activity of these protease inhibitors against PV is comparable to their activity against various strains of HRV, the virus against which the compounds were originally developed (*17*,*35*). Given the excellent oral bioavailability (*35*) and its favorable pharmacokinetic profile, compound 1 may be an attractive candidate for further study for the treatment and prophylaxis of PV infection.

The pirodavir analog R78206 also displayed potent, broad-spectrum activity against PV. As was the case with rupintrivir, pirodavir did not appear to offer sufficient

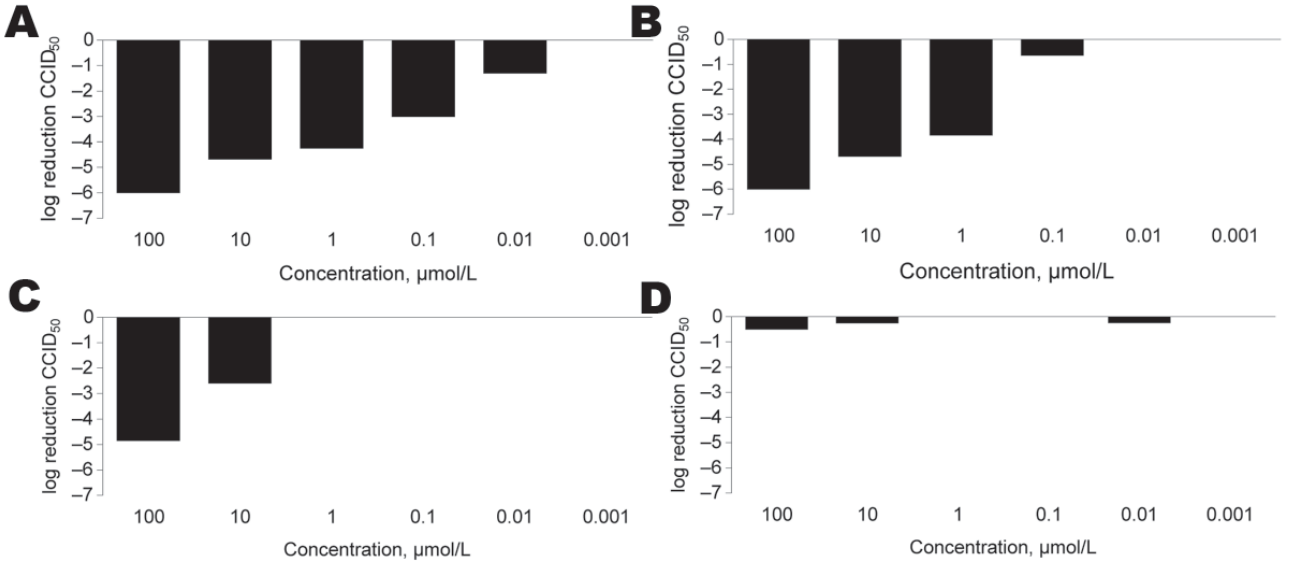

Figure 2. Effect of selected inhibitors on production of infectious poliovirus 1 Sabin in HeLa cell cultures. Supernatants collected from 3 independent experiments were titrated for infectious virus content, and 50% cell culture infective dose ($CCID_{50}$) values were calculated as described by Reed and Muench (*34*). A) Ruprintrivir; B) enviroxime; C) MRL-1237; D) pleconaril.

potential for treating HRV infection and was not further developed. The compound, however, was well tolerated; thus, pirodavir (and its analogs) may, alone or combined with other antiviral agents, open perspectives for treating PV infection. One major problem with pirodavir and its analog, however, is the poor pharmacokinetic profile after systemic dosing due to hydrolysis of the ester bond. Orally bioavailable analogs of pirodavir were developed at Biota (Melbourne, Victoria, Australia) but appeared to have a limited activity toward PV strains (*36*). Another compound that has undergone extensive clinical evaluation for enteroviral infections is pleconaril. The compound is relatively potent in inhibiting PV2 and PV3 replication, but has no activity against PV1, which limits its potential for PV. Besides pleconaril and pirodavir (analog), several other potent capsid-binding agents have been reported (reviewed in [*8*]).

Enviroxime, discovered in 1980, which exhibits potent anti-PV activity, was not developed because of unfavorable pharmacokinetics. However, a further exploration of the potential of enviroxime analogs could be worthwhile, in an attempt to improve the activity, selectivity, and in particular, the pharmacokinetic profile (*25*,*26*).

Compounds that have been less well characterized but that still may form a starting point for the synthesis of more potent and selective inhibitors of PV replication are MRL-1237 and MDL-860. Unraveling the precise mode of antiviral activity and the molecular interaction with their antiviral target may allow structure-based drug design.

Nucleoside polymerase inhibitors that have been developed for treating HCV infection may also have the potential to inhibit other single-stranded positive-sense RNA viruses. Here we demonstrate that the active component of the anti-HCV drug valopicitabine inhibits the replication of all 3 PV strains. If such a drug becomes available for treating HCV infections, it could also be used "off-label" to treat PV infection. However, 4′-azidocytidine, a potent inhibitor of HCV replication (*37*), was devoid of anti-PV activity up to the highest concentrations tested. As reported before and confirmed here, ribavirin proved to be a relatively weak inhibitor of PV replication (TIs >1.8). Although ribavirin has limited activity against HCV when used as monotherapy, its potency is markedly increased when it is given in combination with pegylated interferon. Since extensive clinical experience exists regarding the use of ribavirin in treating HCV infection, it may be possible and beneficial to explore the potential of the combined use of ribavirin with drugs such as rupintrivir, pirodavir, or their analogs.

Because of the high mutation rate of the viral RNA-dependent RNA polymerase, drug-resistant PV mutants have been readily selected in cell culture (*32*,*38*). The possibility that the use of antiviral drugs to treat polio would result in the appearance of drug-resistant variants cannot therefore be excluded. It should be noted, however, that the most potent inhibitors of in vitro PV replication that we identified here (the 3C inhibitors rupintrivir and compound 1, the capsid binders R78206 and pleconaril, and the 3A inhibitor enviroxime), act on different targets in the viral replication cycle. The use in combination of drugs with different modes of action will likely delay or prevent the emergence of drug-resistant variants. Moreover, the period

of treatment during an acute PV outbreak would likely be much shorter than treatment regimens for such chronic infections as HIV or HCV, reducing the chance that drug-resistant strains will emerge.

As highlighted earlier, the need for adequate antiviral drugs against PV (most likely in combination with IPV) in the final stages of polio eradication is obvious. In a recent report from the World Health Organization (*39*), an advisory committee concurred with the proposal to establish a "PV antiviral initiative," to take forward the key recommendations proposed during the National Research Council meeting on antiviral agents against PV.

In the present study, several drugs, some of which have been (rupintrivir, pirodavir, valopicitabine, compound 1) or are being (pleconaril) studied in the clinical setting, are reported to inhibit the in vitro replication of PVs to varying degrees. These drugs, used alone or in combination, may have potential for the treatment or prophylaxis of PV infections. These and other compounds may serve as starting points for the design of more potent PV inhibitors with favorable safety and pharmacokinetic profiles.

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Mr De Palma is a doctoral candidate in the Virology and Experimental Chemotherapy Laboratory at the University of Leuven, Leuven, Belgium. His research focuses on the development of novel antiviral agents against picornaviruses.

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Determination of Oseltamivir Quality by Colorimetric and Liquid Chromatographic Methods

Michael D. Green,* Henry Nettey,* and Robert A. Wirtz*

We developed a colorimetric and chromatographic assay for oseltamivir to assess the authenticity of Tamiflu (F. Hoffmann-La Roche Ltd., Basel, Switzerland) because of a growing concern about counterfeit oseltamivir. The colorimetric assay is quantitative and relies on an extractable colored ion-pair complex of oseltamivir with Congo red or bromochlorophenol blue. The reverse-phase chromatographic assay uses an alkaline mobile phase with UV detection. Both methods were evaluated for variability and selectivity and subsequently applied to batches of oseltamivir products acquired through the Internet. The Congo red test showed greater assay sensitivity, linearity, and accuracy. Colorimetric and chromatographic analysis showed all batches of oseltamivir product were within ±15% of the stated amount of active ingredient.

The antiviral drug oseltamivir phosphate has been recommended by Centers for Disease Control and Prevention as an adjunct in the effective treatment and prevention of influenza. Oseltamivir and zanamivir are both approved by the Food and Drug Administration (FDA) for use in controlling both influenza A and B viruses (*1*). Oseltamivir phosphate is the active ingredient in Tamiflu (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and is available in capsule and powder form.

The specter of an avian influenza pandemic has given Tamiflu much notoriety, and anticipation of the potential public health threat has prompted a demand for the product. Consequently, criminal elements have already begun to produce counterfeit Tamiflu. Recently, US Customs agents seized counterfeit Tamiflu entering the United States (*2*). The bogus products, which contained vitamin C and lacked the active ingredient of oseltamivir phosphate, had been purchased through the Internet. Although these shipments were quickly detected by a joint effort of the FDA and US Customs and Border Protection, these products would easily have gone unnoticed in developing countries, where insufficient resources and infrastructure hamper the ability to monitor and preserve drug quality. WHO estimates that up to 25% of the medicines consumed in developing countries are counterfeit or substandard (*3*).

Simple and affordable colorimetric assays provide a practical means to rapidly monitor drug quality in resource-poor areas. Because oseltamivir phosphate (Figure 1) possesses amine groups, the protonated form may act as a cationic site for anionic dyes such as Congo red and bromochlorophenol blue to produce colored ion-pairing complexes. Congo red has been used in colorimetric determinations of chitosan and poly (*N*-vinyl-2-pyrrolidone) while bromophenol blue has been used in colorimetric assays for antimalarial drugs (*4*–*6*). Therefore, our objective was to develop and evaluate a colorimetric technique, as well as a high-performance liquid chromatographic method (HPLC), to measure the concentration of oseltamivir phosphate in pharmaceutical preparations. The HPLC method described here was used to validate the colorimetric test. To date, there are few published reports of HPLC methods for measuring oseltamivir. A sensitive HPLC-mass spectrometry assay for oseltamivir carboxlate in plasma and urine and an HPLC assay for oseltamivir phosphate in pharmaceutical preparations have been described (*7*,*8*). In our study, we validated, compared, and applied colorimetric and HPLC techniques to the testing of alleged Tamiflu product purchased through the Internet.

Methods

Reagents and Apparatus

All reagents were of analytical-reagent grade, and deionized water was used for all aqueous solutions. Phar-

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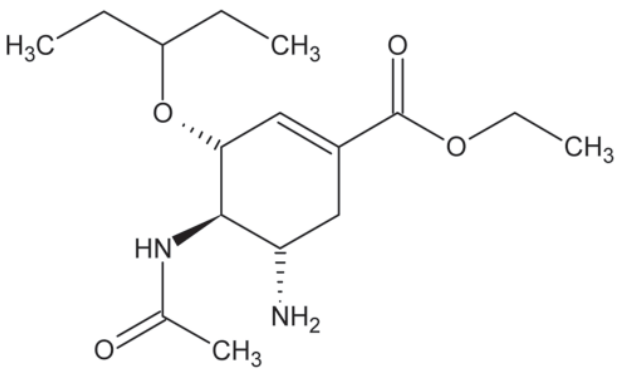

Figure 1. Structure of oseltamivir.

maceutical-grade oseltamivir phosphate was graciously donated by Hoffman-La Roche Ltd. We purchased Congo red (dye content ≈97%), bromochlorophenol blue sodium salt (dye content ≈95%), potassium hydrogen phthalate, monobasic potassium phosphate, sodium bicarbonate, and sodium hydroxide from Sigma-Aldrich (St. Louis, MO, USA); HPLC-grade acetonitrile from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA); and ethyl acetate from Acros Organics (Morris Plains, NJ, USA).

Absorbance measurements were taken using a Spectronic 21 spectrophotometer (Milton Roy, Riviera Beach, FL, USA). HPLC analysis was conducted with an Agilent 1100 Series system (Agilent, Palo Alto, CA, USA) using an X-Terra, RP18, 4.6- × 150-mm column (Waters, Milford, MA, USA).

Sample Preparation

The colorimetric and HPLC methods were evaluated in terms of linearity, assay precision, and accuracy by using pharmaceutical preparations compounded from a mixture of lactose, starch, talc, povidone K30, croscarmellose, and stearyl fumarate, which contained known amounts of oseltamivir phosphate. These inactive ingredients (excipients) are those found in the capsule formulation of Tamiflu (*9*). The excipient mix was kept constant while various ratios of oseltamivir phosphate and lactose were added to produce sample groups containing 0%, 50%, 80%, 100%, 120%, and 150% of the amount of active ingredient normally found in a Tamiflu capsule. The 100% mixture contains 46% of the active ingredient and is equivalent to a capsule containing 75 mg of oseltamivir base (98.5 mg oseltamivir phosphate).

We conducted a search for Tamiflu products on the Internet using the keywords "Tamiflu," "prescription," and "cheap or inexpensive." Approximately 40 online sources were compiled and sorted according to price. We acquired the 6 cheapest products that did not require a prescription and tested for active ingredient by using both colorimetric methods and HPLC. All the products were in capsule form and allegedly contained 75 mg of oseltamivir base (98.5 mg oseltamivir phosphate) as described in the package insert. The contents of the entire capsule were deposited into a glass vial, and 32.8 mL of water (Congo red test) or 8.2 mL (bromochlorophenol blue test) of water was added. The mixture was vigorously shaken for ≈10 s, allowed to equilibrate for 10 min, shaken again, and then filtered through 0.22- or 0.45-μm membranes. The amount of oseltamivir per capsule was then determined by using the colorimetric and HPLC methods.

Colorimetric Assay

Congo red and bromochlorophenol blue salt were evaluated for the colorimetric assay and prepared at a concentration of 1 mg/mL in water. For the Congo red test, a portion of material from each sample group was weighed, and enough water was added to achieve a concentration of 6.5 mg/mL. This is equivalent to 3 mg/mL of oseltamivir phosphate present in the 100% sample group. The filtered sample solution (0.150 mL) was added to a glass siliconized tube containing 0.250 mL of Congo red solution, 0.350 mL of 0.1 M phthalate buffer, pH 4.2, and 3 mL of ethyl acetate. The tubes were capped and the mixture vigorously shaken for 10 s. After complete phase separation, the top organic layer (red, if oseltamivir was present) was transferred to a 13-mm diameter clean glass tube for absorbance measurements at 520 nm. For the bromochlorophenol blue test, the sample was prepared so that the final concentration for the 100% group was 26 mg/mL, which is equivalent to 12 mg/mL of oseltamivir phosphate. The sample was mixed and filtered as described previously, and 0.150 mL was added to a siliconized glass tube containing 0.250 mL of bromochlorophenol blue solution, 0.350 mL of 0.1 M phosphate buffer, pH 7.0, and 3 mL of ethyl acetate. After vigorous mixing and phase separation, the top organic layer (blue, if oseltamivir was present) was transferred to a 13-mm diameter clean glass tube for absorbance measurements at 590 nm. Other drugs commonly used in developing countries, i.e. aspirin, ampicillin, chloroquine, acetaminophen, amoxicillin, ciprofloxacin, quinine, chloramphenicol and erythromycin, were prepared in water at a concentration of 2.5 mg/mL and tested using the described colorimetric conditions.

HPLC Analysis

We used a mobile phase comprising 30% acetonitrile and 70% 0.05 M bicarbonate buffer, pH 10, at a flow rate of 1 mL/min to achieve component separation while maintaining column temperature at 30°C. Oseltamivir was detected by UV absorbance at 254 and 220 nm with a retention time of ≈4 min. Injection volume was 2 μL. The limit of detection was determined from the analyte mass equivalent to 3 times the baseline noise.

Results and Discussion

Colorimetric Assay

Optimal formation of the complex is dependent on the ionization constants (pKa) as well as solubility characteristics for both the basic drug and acidic dye; therefore, optimum complex formation is pH dependent and is characteristic of the analyte being tested. We determined the optimum pH for complex formation between oseltamivir and Congo red to be 4; the optimum pH for bromochlorophenol blue and oseltamivir was 6–7. The absorption spectra for the oseltamivir–Congo red complex (maxima 507 nm) and oseltamivir-bromochlorophenol blue complex (maxima 589 nm) are shown in Figure 2. We evaluated selectivity of the Congo red assay with other commonly used pharmaceuticals. Under the described assay conditions, aspirin, ampicillin, chloroquine, acetaminophen, amoxicillin, ciprofloxacin, and chloramphenicol produced a clear colorless organic phase; quinine and erythromycin showed a very faint rose color. Of the drugs tested for specificity using the bromochlorophenol blue assay, quinine produced a purple color, chloroquine a light blue color, and acetaminophen a faint yellow color. The selectivity of the assay is a function of drug solubility in water as well as pH. Oseltamivir phosphate is highly soluble in water (*9*). Because aspirin, acetaminophen, amoxicillin, quinine, chloramphenicol, and erythromycin are insoluble or slightly soluble in water, most of the material was eliminated by filtration before the assay was conducted and may have contributed to a colorless ethyl acetate phase. Therefore, filtration is considered necessary because aqueous solubility and a pKa confer selectivity of the colorimetric tests with oseltamivir.

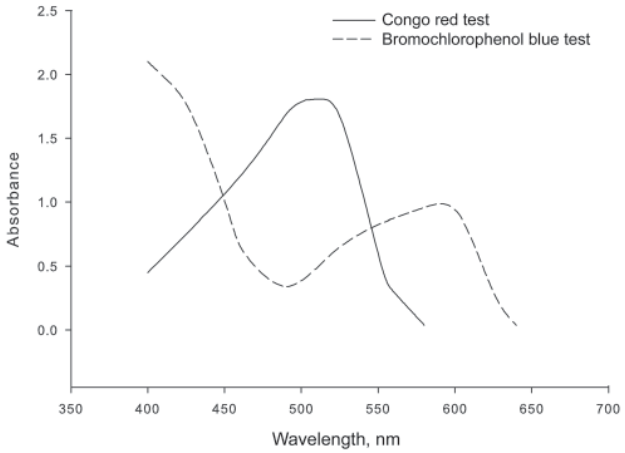

Figure 2. Spectra of Congo red and bromochlorophenol blue complexes with oseltamivir in ethyl acetate.

Evaluation of the colorimetric assay for oseltamivir carboxylate, the active metabolite of the prodrug oseltamivir phosphate (*9*), has not been performed. Ion-pairing with acidic dyes under the described conditions is less likely because the carboxy metabolite is a zwitterion.

Table 1 shows the intraday and interday variability associated with the colorimetric assays; Figure 3 illustrates the linearity of the absorbance versus concentration curve. Greater linearity and lesser variability are observed from the Congo red assay. Note that the variability also includes deviations arising from the preparation of the oseltamivir formulations. The greater slope associated with the Congo red curve relative to the bromochlorophenol blue curve in

Table 1. Accuracy and precision for the high-performance liquid chromatographic (HPLC) and colorimetric assays (n = 5)

	Accuracy, %		Precision, %	
Nominal concentration, mg/mL	Interday	Intraday	Interday	Intraday
HPLC				
0.6	–10.8	–10.3	9.0	7.9
1.5	–8.4	0.4	7.3	2.3
2.4	5.0	–0.7	7.3	5.7
3.0	0.4	1.6	4.9	4.8
3.6	0.7	4.4	4.1	3.6
4.5	–0.9	–3.2	2.5	2.5
Congo red colorimetric				
1.5	2.0	2.2	12.5	2.3
2.4	2.6	–0.3	9.2	6.9
3.0	–5.5	1.0	6.5	5.4
3.6	0.7	1.2	3.7	3.8
4.5	1.0	–0.1	2.6	1.3
Bromochlorophenol blue colorimetric				
6.0	–5.3	–19.4	18.6	12.4
9.6	0.0	3.4	13.5	2.6
12.0	2.7	10.4	6.1	5.9
14.4	3.5	3.2	4.3	3.8
18.0	–2.9	–5.4	4.9	3.0

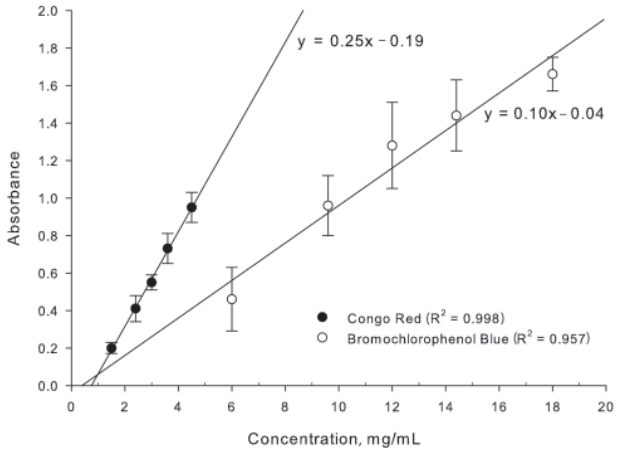

Figure 3. Linearity of colorimetric assays.

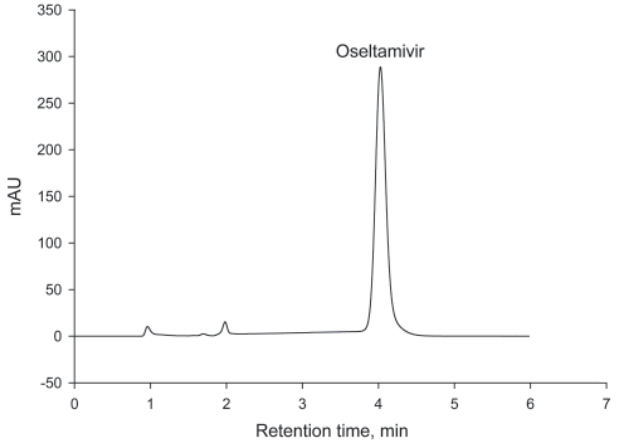

Figure 4. Chromatogram of oseltamivir from Tamiflu purchased over the Internet.

Figure 3 demonstrates a more sensitive assay. The results of the colorimetric assays for the oseltamivir phosphate products purchased over the Internet are shown in Table 2 and compared with values determined from HPLC analysis. These values are the percentage of active ingredient found per capsule relative to that stated on the manufacturer's package insert. All, except brand B (Cipla), were Roche brand products. The senders' addresses for brands C, D, and E were all within the United States, while brand C originated from India and brands A and F originated from Greece. Except for brand B, all products were within ±10% of the stated amount of active ingredient.

HPLC Analysis

Intraday and interday accuracy and precision were within ±11% for oseltamivir phosphate concentrations of 0.6 mg/mL to 4.5 mg/mL (Table 1). Mobile phase pH above the pKa of a basic analyte generally produces chromatograms with a good symmetrical peak shape. The chromatogram for oseltamivir is shown in Figure 4. Because the pKa of oseltamivir is 7.75 (*9*), a mobile phase comprising a pH 10 (2 U above the pKa) bicarbonate buffer was chosen. The C18 column used for the HPLC method is designed to operate under basic pH conditions. Injections of aqueous mixtures of aspirin, ampicillin, chloroquine, acetaminophen, amoxicillin, ciprofloxacin, quinine, chloramphenicol, or erythromycin into the HPLC system showed no interfering chromatographic peaks. The limit of detection for oseltamivir phosphate at 220-nm and 254-nm detection wavelengths are 2.2 ng and 4.2 ng, respectively.

Table 2. Evaluation of oseltamivir products purchased over the Internet*

Brand	HPLC	Congo red	Bromochlorophenol blue
A	94 ± 1	95 ± 3	106 ± 4
B	87 ± 2	88 ± 3	97 ± 8
C	94 ± 4	93 ± 2	103 ± 3
D	96 ± 1	93 ± 5	107 ± 7
E	97 ± 3	89 ± 3	104 ± 5
F	95 ± 0	88 ± 2	101 ± 3

*HPLC, high-performance liquid chromatography. Values are the percentage of active ingredient found per capsule relative to that stated on the manufacturer's package insert (average ± SD; n = 3 capsules).

Conclusions

Anionic dyes such as Congo red and bromochlorophenol blue form colored ion-pairing complexes with oseltamivir to produce a colored product extractable in ethyl acetate. The Congo red method produces a colored product, which is more linearly proportional to oseltamivir concentration, has less variability, and is more selective than the bromochlorophenol blue method.

Colorimetric tests are rapid and easy to perform. The reagents and equipment for colorimetric tests are inexpensive, relatively nontoxic, and are ideal for use in field situations.

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We thank Zakia al-Amin and Melissa Fox for their contributions to the project.

Dr Green is a chemist in the Division of Parasitic Diseases, Centers for Disease Control and Prevention. His research interests include developing low-cost field-adapted techniques for rapid drug quality evaluations, developing high-performance chromatographic methods for antimalarial drug analysis, and performing pharmacokinetic studies of antimalarial drugs.

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Clonal Population of Flucytosine-Resistant *Candida tropicalis* from Blood Cultures, Paris, France

Marie Desnos-Ollivier,* Stéphane Bretagne,*† Claire Bernède,*‡ Vincent Robert,§ Dorothée Raoux,* Elisabeth Chachaty,¶ Elisabeth Forget,# Claire Lacroix, Françoise Dromer,* and the YEASTS group[1]**

Candida tropicalis is a diploid ascomycetes yeast responsible for 4%–24% of candidemia. Resistance to flucytosine is rarely described for this species but was observed for 45 (35%) of 130 *C. tropicalis* isolates recovered from blood cultures in the Paris area in a 4-year survey. The aims of this study were to test the hypothesis that the flucytosine-resistant isolates could represent a subgroup and to determine the relationship between epidemiologic and genomic data. Epidemiologic data and gene sequences were analyzed, and molecular typing was performed. Our results suggest that a clone of flucytosine-resistant isolates, associated with malignancies and a lower mortality than that for other *C. tropicalis* isolates, is widespread in the Paris area. We propose the analysis of 2 polymorphic microsatellite markers coupled with *URA3* sequencing to track the clone.

Candida tropicalis is a diploid ascomycetes yeast commonly found on the skin and in digestive tracts of healthy human hosts worldwide (*1*). Infections caused by *C. tropicalis* are reported in 4%–24% of patients with candidemia, depending on the country of study, underlying risk factors, and period of study (*2*). Primary resistance to flucytosine (5FC) occurs in <5% of all *Candida* species except for *C. krusei*, in which it is detected in up to 28% of isolates (*3*). It was thus unexpected to observe 35% resistance to 5FC among *C. tropicalis* isolates recovered from blood cultures in the active surveillance program on yeast-related fungemia implemented by the French National Reference Center for Mycoses and Antifungals (NRCMA) in the Paris area. The YEASTS program is designed to analyze the epidemiologic trends of yeast fungemia by collecting isolates and epidemiologic and clinical data. The second objective is to study the clinical isolates in terms of species, antifungal susceptibility profiles, and genetic diversity to look for associations between subtypes of isolates and epidemiologic/clinical parameters. To test the hypothesis that the 5FC resistant ($_R$5FC) isolates could represent a different species or a subgroup, the $_R$5FC and susceptible ($_S$5FC)

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isolates were compared on the basis of several phenotypic and molecular features.

Materials and Methods

Strains

Clinical isolates of *C. tropicalis* recovered from blood cultures during the YEASTS program from October 1, 2002, through September 30, 2006, were selected for the study. Epidemiologic and clinical data concerning the patients were collected by using a standardized electronic form. Isolates (1 isolate/patient) were sent to NRCMA for identification and MIC determination (see below). All isolates were stored frozen in 40% glycerol at –80°C.

The type strain of *C. tropicalis* CBS 94 (ATCC 750, $_S$5FC) was included in the study as a reference. In addition, 29 strains of taxonomic synonyms available at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands) were studied.

Phenotypic Characterization of All *C. tropicalis* Isolates

All isolates were identified at the species level by using the assimilation patterns obtained with the commercialized strips ID32C (bioMérieux, Marcy-l'Etoile, France). MICs to 9 systemic antifungal agents were determined for all clinical isolates and the type strain by using the EUCAST microdilution method (*4*). For nonclinical isolates, only MICs of 5FC and fluconazole were determined.

Additional Studies on Selected Isolates

Growth Characteristics

For the first 16 $_S$5FC and 14 $_R$5FC consecutive isolates of *C. tropicalis* and for CBS 94 other studies were performed. Additional carbon sources were tested by using the commercial strips CH50 (bioMérieux). Maximal temperature of growth (42°C or 45°C) was determined on Sabouraud dextrose agar. Growth in hyperosmolar medium (50% glucose or 10% NaCl) was also evaluated.

Nucleotide Sequence Determination

After 24 hours of incubation at 27°C on Sabouraud agar plates, single colonies were discharged in 1 mL of distilled water in a microcentrifuge tube, and DNA extraction was performed by using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. Universal fungal primers were used for the amplification of the internal transcribed spacer 1 (ITS1)–5.8S-ITS2 (primers V9D and LS266 [*5*,*6*]) and the 26S (primers NL1 and NL4 [*7*]) rDNA regions. Primers (Table 1) were designed to amplify a partial sequence of the actin gene (GenBank accession nos. AJ389059 and AJ508499) and the 14-α-demethylase gene (GenBank accession nos. AY942646 and M23673). Reaction volumes of 20 μL contained 1 μL of genomic DNA, 1.25 U of AmpliTaq Gold, 2 μL of PCR buffer 10×, 2 μL of 25 mmol/L $MgCl_2$, 2 μL of 2.5 mmol/L deoxyribonucleoside triphosphates (dNTPs) (Roche), and 1 μL

Table 1. Primers sequences and amplification parameters used in the present study

Locus	Primer	5′ labeling	Sequence (5′ → 3′)*	Annealing temperature, °C
14αDM†	DMC1		>TGGGTGGTCAACATACTTC	50
	DMC2‡		<CATCTRTGTCTACCACCACC	
Actin	ACTa		>AAGGTATTATGGTTGGTATGG	55
	ACTb		<TCGAAATCTAAAGCAACGTAA	
URA3	URAF	HEX§	>ATTGGATAGTCCCTCTAAACTCACTACTA	55
	URAr		<AGCATTAGTTATATCACTCCACGATGAA	
	URAF2		>TGCCGATATTGGAAATACAGTTA	50
	URAr2		<AATCAACTATTCAAGTTGACCG	
	CTU2		<GTTGGAACATCAATTGATGCACATAAAT	55
Unknown	CT14a	6FAM¶	>GTAAATCTTGTATACCGTGGA	55
	CT14b		<TAGCCCATTTTCTAGTTTTGC	
FCY1	CTCDf		>ATCATTAGTTCAGATGGTAAAGTCTTG	58
	CTCDr		<CCTTTTTAGTAACATGTCTATTCTCCA	
FCY2	CTCP1f		>TGCCCATAAATTAAATGCAGAA	58
	CTCP1r		<GGAAGCAACAAACCCAAAAA	
FCY2	CTCP2f		>TGCTGCCGATTATGTTGTTT	58
	CTCP2r		<GTGAAAACGAGCCAATCCAT	
FUR1	FUR1f		>TCATCAAAACCATGTCTGCTG	58
	FUR1r		<AAGTGTATGTAGTGATAATTGCTATGC	

*>, Sense primer; <, antisense primer.
†14 α demethylase.
‡R = G or A.
§4, 7, 2′,4′, 5′,7′-hexachloro-6-carboxyfluorescein.
¶6-carboxyfluorescein.

(10 μM) of primers. The PCR products were amplified by using the ICycler Thermocycler (Bio-Rad, Marnes-La-Coquette, France) set up with a first cycle of denaturation for 10 min at 95°C, followed by 30 cycles of denaturation at 94°C for 30 s, 30 s at the relevant annealing temperature, elongation at 72°C for 30 s, and a final extension step of 10 min at 72°C. Both strands of purified amplified fragments were sequenced at the Genopole of the Pasteur Institute, on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Courtaboeuf, France), with the same primers that were used in the PCR step. Sequences were edited with Chromas Pro version 1.33 (Technelysium Pty Ltd, Helensvale, Queensland, Australia). Multiple sequences alignments were performed with ClustalW version 1.8 (www.ebi.ac.uk/clustalw).

Following preliminary results, primers were then designed to amplify the complete sequence of the orotidine-5′-phosphate decarboxylase gene (URA3, GenBank accession no. AF040702) (Table 1). Furthermore, the complete sequences of *FCY1* (coding for the cytosine deaminase), *FCY2* (coding for the purine cytosine permease), and *FUR1* (coding for the uracil phosphoribosyl transferase) were determined. Primers were designed by using sequences from the Broad Institute *C. tropicalis* database genome (locus CTRG_02927.3 for *FCY1*, locus CTRG_02059.3 for *FCY2*, and locus CTRG_02689.3 for *FUR1*) (Table 1). The sequences were amplified as described above (except for the duration of annealing and elongation [1 min] when using the primers set CTCP1f/CTCP1r). The sequences were translated with the standard genetic code (www.bioinformatics.org/sms/index.html). The resulting protein sequences were aligned with the BioloMICS software (BioloMICS, version 7.2.5, BioAware S.A., Hannut, Belgium).

Microsatellite Selection

C. tropicalis genome sequences available from GenBank databases and from the Broad Institute (www.broad.mit.edu/annotation/fungi/candida_tropicalis) were studied to identify sequences containing microsatellite repeats. Two polymorphic microsatellite markers (PMMs) were selected, 1 upstream of the *URA3* gene (URA3 PMM) and 1 on a nonannotated sequence (CT14 PMM). Oligonucleotide primers were designed from the sequence of the corresponding flanking regions to obtain PCR products ranging in size from 100 bp to 200 bp. One primer of each set was 5′ labeled with different dyes (Table 1). PCR was conducted independently for the 2 loci in a 20-μL reaction volume containing 2 μL of extracted DNA, 1.25 U of AmpliTaq Gold, 2 μL of PCR Buffer 10×, 4 μL of 25 mmol/L $MgCl_2$, 2 μL of 2 mmol/L dNTPs, and 0.2 μL (10 μM) of primers. PCR amplifications were performed for a total of 27 cycles by using the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension step of 5 min at 72°C. Two microliters of each PCR product mixed with 20 μL of formamide and 0.5 μL of an internal standard labeled with 6-carboxy-X-rhodamine dye (GeneScan-500 Tamra, Applied Biosystems) was run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sizes of the allele and PCR fragments were determined with GenScan 3.0 (Applied Biosystems, Weiterstadt, Germany). To assign a specific length to a PCR fragment, all electromorphs were aligned with that of the type strain (CBS 94). Each allele was named after the length of PCR fragments. Isolates for which 1 signal was observed for a given locus on the electromorph were considered homozygous for this locus by analogy with what is reported for another diploid yeast, *C. albicans* (*8*).

Multilocus Sequence Typing Analysis

Three of the 6 MLST loci recently described were analyzed as reported in Tavanti et al. (*9*). These loci were selected because, according to these authors, they were associated with more polymorphism (XYR1 and SAPT4) and with antifungal resistance (MDR1). Both strands of purified amplified fragments were sequenced, and sequences were edited as described above. Heterozygosity was defined by the presence of 2 coincident peaks of similar height in the forward and the reverse sequence chromatograms. The 1-letter code for nucleotides from the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC, www.bioinformatics.org/sms/iupac.html) was used. Sequences were compared with the allele sequences of the *C. tropicalis* MLST database (www.pubmlst.org/ctropicalis). For each gene, distinct alleles were identified and numbered by using the Internet-based MLST program (www.mlst.net). New alleles were submitted to the MLST *C. tropicalis* database.

Statistical Analysis

In accordance with French regulations, the clinical database was approved by the Commission Nationale de l'Informatique et des Libertés. Information concerning demographic data, risk factors for candidiasis, and outcome 30 days after the diagnosis of fungemia were recorded. We considered 3 groups of patients according to the infecting isolate: $_S$5FC, $_R$5FC that belong to the clone ($_R$5FC clone, see below), and $_R$5FC that do not belong to the clone (termed "other $_R$5FC"). The sociodemographic and clinical characteristics were compared between the 3 groups of isolates by using the Fisher exact test. The χ^2 Armitage trend test (*10*) was used to assess a trend in the evolution of the $_R$5FC clone's proportion among resistant strains across years of study. Multinomial logistic regression (*11*) adjusted on clinical center was used to investigate the factors associated with infection by the $_R$5FC clone or other $_R$5FC

isolates compared to $_S$5FC isolates according to sociodemographic and clinical characteristics. A logistic regression model adjusted on clinical center was also performed to identify the factors associated with the acquisition of the $_R$5FC clone compared with other $_R$5FC isolates. Regression models were constructed by using the backward procedure. First, all covariates with a p value <0.25 in univariate models were simultaneously entered into the regression model. The set of covariates with the largest p value was iteratively removed from the model until all of the covariates (or blocks of covariates) remaining in the reduced model had a p value <0.05. Statistical analyses were performed with Stata software, version 9.0 (StataCorp, College Station, TX, USA).

Results

Phenotypic Characterization of $_R$5FC Isolates

We analyzed the episodes of fungemia caused by *C. tropicalis* and recorded during the first 4 years of the YEASTS study; 130 episodes were recorded in 24 of the 27 participating centers. Distribution of flucytosine MICs showed 2 populations, 1 with MICs <2 μg/mL and 1 with MICs ≥8 μg/mL (Figure). In light of these results, susceptibility to 5FC ($_S$5FC) was defined by an MIC <8 μg/mL and resistance ($_R$5FC) by an MIC ≥8 μg/mL.

The proportion of $_R$5FC isolates (45 [35%]) of the 130 isolates) was uneven, ranging from 0% to 67% of the isolates, depending on the center of isolation. However, the proportion of $_R$5FC isolates did not differ over the study period (data not shown). We first studied the characteristics of a subset of 16 $_S$5FC and 14 $_R$5FC *C. tropicalis* isolates (strain CBS 94 had all the characteristics of $_S$5FC clinical isolates described below but is not included in the analysis). There was no difference in terms of growth in hyperosmolar media between $_R$5FC and $_S$5FC clinical isolates. By contrast, $_R$5FC and $_S$5FC isolates differed in the proportion of isolates growing at 45°C (40% vs. 100%, p<0.001) and assimilating starch (12.5% vs. 50%, p = 0.054) and xylitol (62.5% vs. 12.5%, p = 0.009). No difference in the MIC of azoles or caspofungin was noted. All 29 strains of *C. tropicalis* synonyms stored at the CBS exhibited 5FC MICs ≤0.5 μg/mL.

Genotypic Characterization of $_R$5FC Isolates

This subset of isolates (16 $_S$5FC and 14 $_R$5FC) was further analyzed. The deletion of 1 nucleotide (A) in position 106 (according to the type strain sequence, GenBank accession no. AY939810) of the ITS2 region was observed in 14 (100%) of the 14 $_R$5FC isolates compared to 5 (32%) of the 16 $_S$5FC isolates (p<0.001) (GenBank accession no. EU288196). No difference in nucleotide sequences was found for the D1/D2 region of the 26S rDNA

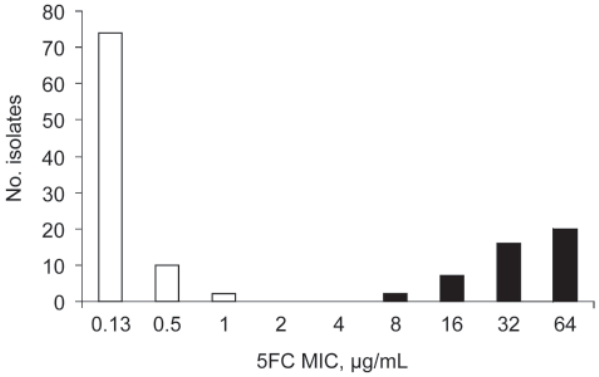

Figure. Distribution of 130 *Candida tropicalis* isolates recovered from blood cultures during the first 4 years of an active surveillance program (YEASTS study) on yeasts fungemia in the Paris area, France (October 2002 through September 2006), according to the MICs of flucytosine determined with the EUCAST microdilution method (*4*).

or in the portion of the 14-α demethylase (490 bp) and actin (550 bp) genes analyzed. PMM results are summarized in Table 2. The URA3 and CT14 PMM led to 6 and 7 different allelic associations, respectively. The association of both markers led to 13 PMM profiles. The 14 $_R$5FC isolates had the same URA3/CT14 PMM profile; however, among the 16 $_S$5FC, 15 had a PMM profile different from that of the $_R$5FC, and 1 (ODL6–560) had the $_R$5FC PMM profile. All but 1 (ODL2–237) of the $_R$5FC isolates had the same MLST profile, whereas none of the $_S$5FC isolates exhibited the same combination of the 3 MLST studied. The entire *URA3* nucleotidic sequence of the translated region was identical except in 1 position (GenBank accession no. EU288194 for the type strain CBS 94 and EU288195 for 1 of the $_R$5FC isolates). $_S$5FC isolates were either homozygous or heterozygous at position 529 (A-A or A-G), whereas all the $_R$5FC isolates were homozygous G-G. This produced a change of K177E (lysine → glutamate) for the $_R$5FC isolates.

Thus, results obtained with nucleotide changes (deletion of A in position 106 in the ITS2 region, mutation in position 529 of the *URA3* gene) and polymorphisms in 3 MLST and 2 PMMs suggested that the 14 $_R$5FC studied were clonal. We thus decided to use the 2 PMMs (URA3 PMM, CT14 PMM) to genotype all the *C. tropicalis* isolates recovered during the study period in the YEASTS program. Of the 130 *C. tropicalis* isolates (including the 30 isolates studied above), 45 were $_R$5FC (Table 3). Thirty-three different profiles were observed when both PMMs were combined for the 130 isolates. Among the 45 $_R$5FC, a total of 29 isolates exhibited the profile associated with the $_R$5FC clone; 16 were different, with 11 different profiles, 6 of which were shared with $_S$5FC isolates. Among the $_S$5FC

Table 2. Comprehensive analysis of 30 *Candida tropicalis* isolates*

Strain no.	5FC MIC (μg/mL)	Month of isolation	SNP ITS2	PMM alleles		MLST			*URA3* base 529
				URA3	CT14	MDR1	XYR1	SAPT4	
ODL1–18	>64	2002 Nov	–	178/178	148/151	20	26	10	G
ODL1–40	>64	2002 Nov	–	178/178	148/151	20	26	10	G
ODL1–41	>64	2002 Nov	–	178/178	148/151	20	26	10	G
ODL1–53	16	2002 Dec	–	178/178	148/151	20	26	10	G
ODL2–105	>64	2003 Mar	–	178/178	148/151	20	26	10	G
ODL2–198	32	2003 Jul	–	178/178	148/151	20	26	10	G
ODL2–199	>64	2003 Jul	–	178/178	148/151	20	26	10	G
ODL3–237	>64	2003 Sep	–	178/178	148/151	18	26	10	G
ODL3–293	>64	2003 Oct	–	178/178	148/151	20	26	10	G
ODL4–311	>64	2003 Sep	–	178/178	148/151	20	26	10	G
ODL4–328	>64	2003 Nov	–	178/178	148/151	20	26	10	G
ODL4–341	>64	2003 Nov	–	178/178	148/151	20	26	10	G
ODL5–426	>64	2003 Dec	–	178/178	148/151	20	26	10	G
ODL6–558	32	2004 Apr	–	178/178	148/151	20	26	10	G
ODL1–58	<0.125	2003 Jan	+	176/176	148/148	24	30	7	A
ODL3–211	0.25	2003 Jul	+	174/178	148/148	1	**79**	1	A-G†
ODL3–231	<0.125	2003 Sep	–	176/176	151/151	**66**	9	18	A
ODL4–302	0.25	2003 Sep	–	176/178	148/151	4	36	23	A
ODL4–347	0.25	2003 Nov	+	174/174	151/154	7	52	6	A
ODL4–384	0.5	2003 Dec	–	174/176	151/151	**67**	4	19	A
ODL5–460	<0.125	2004 Feb	+	174/178	148/148	**68**	**76**	36	A-G†
ODL5–474	<0.125	2004 Mar	+	174/174	154/154	7	52	6	A
ODL5–476	<0.125	2004 Mar	+	178/178	151/151	27	4	11	A
ODL5–485	<0.125	2004 Mar	–	176/178	148/157	22	41	7	A
ODL5–488	<0.125	2004 Apr	+	176/178	148/151	25	24	7	A
ODL6–504	<0.125	2004 Feb	+	174/178	148/151	22	9	38	A
ODL6–511	<0.125	2004 Apr	+	174/178	148/148	1	**80**	1	A-G†
ODL6–521	<0.125	2004 May	+	176/178	148/151	**69**	**77**	**41**	A
ODL6–539	<0.125	2004 Mar	+	174/174	148/154	58	48	13	A
ODL6–560	<0.125	2004 Jul	–	178/178	148/151	4	36	23	A
CBS94	<0.125	–	+	176/176	148/148	**70**	**78**	5	A

*5FC, flucytosine; SNP, single nucleotide polymorphism; ITS2, internal transcribed spacer 2; PMM, polymorphic microsatellites marker; MLST, multilocus sequence typing (**boldface** corresponds to new alleles).
†A-G heterozygous.

isolates, 4 had the PMM profile associated with the $_{R}$5FC clone. The *URA3* gene was sequenced for these 4 isolates, and none had a G in position 529.

We then studied genes potentially involved in the mechanisms of 5FC resistance. For the FUR1 sequences, no missense mutation was observed, and the complete coding sequence of the type strain CBS 94 (GenBank accession no. EU327978) was similar to the sequence of the $_{R}$5FC clone; however, a few silent mutations were observed in a few $_{S}$5FC isolates (GenBank accession nos. EU327979, EU327980, and EU327981). Concerning the cytosine deaminase sequences (FCY1), only 1 silent mutation, C21T, occurred for the $_{R}$5FC clone (GenBank accession no. EU327982). Finally, for the purine cytosine permease (FCY2), the sequences of the type strain (GenBank accession no. EU327983) and of the $_{R}$5FC clone were similar. A few heterozygosities were observed for the $_{S}$5FC isolates (GenBank accession nos. EU327984 and EU327985), but all these mutations were silent.

Factors Associated with Fungemia Caused by the *C. tropicalis* $_{R}$5FC Clone

All 130 isolates corresponded to incident fungemia in different persons. The $_{R}$5FC clone was recovered during the 4 years of study with a trend toward a decreased proportion over time (11/13 [85%], 6/10 [60%], 8/13 [61.5%], and 4/9 [44%]) during the first, second, third, and fourth year of the study, respectively; p = 0.06). The proportion of the clone also varied across clinical centers (data not shown). Factors associated with fungemia caused by the $_{R}$5FC clone of *C. tropicalis* were analyzed (Table 4). The proportion of patients infected by the $_{R}$5FC clone was significantly higher among patients with malignancies but their death rate was significantly lower than for patients infection with other $_{R}$5FC or $_{S}$5FC isolates. Multinomial logistic regression was adjusted by clinical centers to investigate the factors associated with infection by the $_{R}$5FC clone or others $_{R}$5FC isolates compared with $_{S}$5FC isolates. The risk of being infected by the $_{R}$5FC clone compared with a $_{S}$5FC isolate significantly increased in case of malignancy (odds

Table 3. Distribution of the polymorphic microsatellites markers (PMM) profiles among 130 *Candida tropicalis* isolates, according to their susceptibility to flucytosine (5FC)*

Allelic association		Total no. isolates	No. isolate types		
URA3 PMM	CT14 PMM	(N = 130)	$_S$5FC (n = 85)	$_R$5FC clone (n = 29)	Other $_R$5FC (n = 16)
172/172	142/148	3	3	–	–
172/172	148/154	2	2	–	–
172/174	142/148	3	2	–	1
172/174	148/148	1	1	–	–
172/174	148/154	1	1	–	–
172/176	142/148	5	4	–	1
174/174	142/148	5	3	–	2
174/174	148/148	1	–	–	1
174/174	148/151	1	1	–	–
174/174	148/154	6	6	–	–
174/174	151/154	1	1	–	–
174/174	154/154	1	1	–	–
174/176	142/148	6	6	–	–
174/176	148/151	1	1	–	–
174/176	151/151	3	3	–	–
174/178	142/148	8	5	–	3
174/178	145/151	1	–	–	1
174/178	148/148	5	5	–	–
174/178	148/151	2	1	–	1
176/176	142/148	11	10	–	1
176/176	148/148	3	3	–	–
176/176	148/151	4	4	–	–
176/176	151/151	1	1	–	–
176/178	142/148	3	3	–	–
176/178	145/151	1	1	–	–
176/178	148/148	1	–	–	1
176/178	148/151	5	5	–	–
176/178	148/157	1	1	–	–
176/180	151/151	1	1	–	–
178/178	142/148	3	2	–	1
178/178	145/151	6	3	–	3
178/178	148/151	33	4	29	–
178/178	151/151	1	1	–	–

*S subscript, susceptible; R subscript, resistant.

ratio [OR] 3.7, 95% confidence interval [CI] 1.4–10.1, p = 0.009), and the risk for death at day 30 after fungemia was significantly decreased in patients infected by the $_R$5FC clone compared with the $_S$5FC isolates (OR 0.3, CI 0.1–0.9, p = 0.04), while no independent factor accounted for infection by other $_R$5FC isolates versus an $_S$5FC isolate. The only independent factor associated with infection by the $_R$5FC clone compared with other $_R$5FC isolates was the death rate at day 30 (OR 0.1, CI 0.03–0.6, p = 0.006).

Discussion

The bimodal distribution of 5FC MICs against *C. tropicalis* isolates prospectively collected from 27 different clinical centers in the Paris area (YEASTS program, Figure) suggested that the *C. tropicalis* population was heterogenous. On the basis of physiologic characteristics and molecular analysis (nucleotide sequences of the ITS regions, D1/D2 region of the large subunit, and large portions of the actin and 14-α-demethylase genes showing >99% similarity), we first assessed a subset of isolates (the first consecutive 14 $_R$5FC and 16 $_S$5FC isolates) and determined that both populations belong to the same species.

All 14 $_R$5FC isolates had a single nucleotide deletion in position 106 of the ITS2 region, although 5 of the 16 $_S$5FC isolates harbored it. When additional genotypic markers were used, all 14 $_R$5FC isolates had the same allelic combination for 2 PMMs selected (URA3 and CT14), the same missense mutation in the *URA3* gene, and the same diploid sequences for the 3 MLST loci studied. By contrast, only 1 of 16 $_S$5FC isolates had the same PMM profiles as the $_R$5FC isolates, but this isolate differed in its MLST profile and the lack of mutation in the *URA3* gene. In addition, the 16 $_S$5FC isolates exhibited 16 different MLST and 12 different PMM profiles. This finding suggested the existence of a $_R$5FC clone, but the rest of the population was genetically diverse. We thus analyzed the 130 isolates of *C. tropicalis* collected over 4 years in the YEASTS program by using the 2 PMMs and sequenced the *URA3* gene when the PMM

Table 4. Patient characteristics according to the 3 categories delineated by the susceptibility of the *Candida tropicalis* isolates to flucytosine (5FC) and their belonging to the $_R$5FC clone*

Characteristic	$_S$5FC (n = 85)	$_R$5FC clone (n = 29)	Other $_R$5FC (n = 16)	p value†
≥60 y of age	39 (46)	17 (59)	9 (56)	0.452
Male	55 (65)	18 (62)	10 (63)	0.962
Had malignancies	41 (48)	22 (76)	9 (56)	0.033
Cancerous	15 (18)	7 (24)	5 (31)	0.374
Hematologic	26 (31)	15 (52)	4 (25)	0.082
In intensive care unit	44 (52)	12 (41)	4 (25)	0.116
Had central venous catheter	68 (80)	24 (83)	12 (75)	0.894
Had recent surgery	27 (32)	8 (30)	4 (25)	0.918
Had prior antifungal therapy	11 (13)	0	2 (13)	0.093
Died before day 30	34/81 (42)	6/28 (21)	10/15 (67)	0.014

*S subscript, susceptible; R subscript, resistant. Values are no. (%).
†Fisher exact test.

profile was identical to that of the 14 $_R$5FC isolates previously studied. We discovered that 29 (64%) of 45 $_R$5FC isolates had an identical PMM profile ($_R$5FC clone), while 11 and 30 different PMM profiles were found among the other 16 $_R$5FC isolates and the 85 $_S$5FC isolates, respectively. According to these data, we assumed that these 29 $_R$5FC isolates were clonal or at least highly genetically related.

The proportion of 35% of *C. tropicalis* isolates resistant to 5FC is unusual (*3*). Other studies report between 0 (*12*) and 15% (*13*) with intermediate values (*14–16*), and all the isolates stored as *C. tropicalis* in the CBS collection since 1912 exhibited 5FC MIC ≤0.5 μg/mL. When we started the YEASTS program in 2002, the proportion of $_R$5FC was already at 46% and the clone accounted for 85% of the $_R$5FC isolates. The trend test suggested that the dispersal of the clone is declining in the Paris area. Whether this decline is specific for blood isolates or is a geographically and temporally restricted phenomenon deserves evaluation by using isolates collected over time from various body sites and geographic areas. An old report on isolates collected from various regions of France established with a nonstandardized technique that as many as 70% of the 63 isolates tested had an MIC ≥32 μg/mL in the 1980s (*17*), and a recent study from Germany on clinical isolates recovered from various body sites including blood reported that 58.3% of isolates were resistant (*18*). Whether any of these isolates belong to the $_R$5FC clone would be of interest. Of note, a recent study of 104 *C. tropicalis* clinical isolates recovered from various countries (*9*) showed that none of the 5 $_R$5FC isolates collected in the United Kingdom has the MLST profile of the $_R$5FC clone.

In the univariate analysis, patients infected by the $_S$5FC or $_R$5FC isolates or by the $_R$5FC clone differed significantly in terms of proportion of underlying malignancies (higher in patients with the clone) and death rate 30 days after fungemia (lower for patients infected by the clone). The $_R$5FC isolates as a whole, and the $_R$5FC clone specifically, were unevenly distributed around the Paris area. When adjusted for clinical center, logistic regression analysis showed that, compared to infection by $_S$5FC isolates, no factor was independently associated with infection by $_R$5FC isolates other than the clone, whereas 2 parameters were associated with infection by the clone. Indeed, malignancies multiplied the risk of being infected by the clone by almost 4 and the risk for death was divided by 3 in case of infection with the clone. *C. tropicalis* fungemia, independent of susceptibility to flucytosine, has already been associated with hematologic malignancies (*19*,*20*) (unpub. data from the YEASTS group). Whether the $_R$5FC clone is less virulent, as established for *C. albicans* isolates with decreased susceptibility to 5FC, remains to be determined (*21*).

The resistance to 5FC was associated with the K177E mutation in the *URA3* gene in the clone. The mechanism of 5FC action is a consequence of intrafungal formation of 2 metabolites, 5-fluorodeoxyuridine monophosphate and 5-fluorouridine triphosphate, which alter DNA and protein synthesis (*22*). The URA3 enzyme (orotidine 5′-phosphate decarboxylase, ODCase) is involved in the metabolic pathway of uridyl-monophosphate (UMP), which is a substrate of thymidylate synthetase and UMP kinase, both involved in nucleic acid synthesis. This mutation involves an amino acid already known to be variable among reference strains (e.g., ATCC 20336), but it has not been associated with modification of the URA3 properties thus far (T. Noël, pers. comm.). Nevertheless, this mutation could, for example, modify the tridimensional structure of the protein, thereby affecting the binding affinity of the substrate for the catalytic site and thus modifying the ODCase efficacy. The ODCase is not known to interfere directly with 5FC activity. However, one of the resistance mechanisms against 5FC consists in increasing the transcription of all the genes involved in the de novo pyrimidine biosynthetic pathway (including *URA3*) to overproduce UMP (*23*).

The K177E mutation is associated with a specific PMM upstream the gene, with a possible role in the level of transcription. The fact that this PMM is homozygous may be due to a loss of heterozygosity. This phenomenon has been recently reported for *C. albicans* and the resistance

to azoles (*24*) and for a specific *C. albicans* isolate and the resistance to caspofungin (*25*).

The mutations described in the 5FC resistance of *C. albicans* (*26*) or *C. lusitaniae* (*27*) involved 3 major genes: *FCY2* coding for the purine cytosine permease, which enables 5FC to enter the fungal cell; *FCY1* coding for the cytosine deaminase, which transforms 5FC into 5FU; and *FUR1* coding for the uracil phosphoribosyl transferase, which transforms 5FU into 5FUMP. The fact that the clone was susceptible to 5FU suggests that the 5FC resistance could result from a mutation in the cytosine deaminase, the cytosine permease, or both (*23*). However, the sequences of the $_{R}$5FC clone, some $_{S}$5FC isolates, and the type strain CBS94 did not show any mutation in coding sequences of *FCY1*, *FCY2*, or *FUR1* susceptible to explain the resistance of the clone to 5FC. The mechanism explaining the possible relationship between the specific PMM (URA3 178/178 and CT14 148/151), the K177E mutation, and the resistance to 5FC remains to be determined.

Our results suggest that a clone of $_{R}$5FC isolates responsible for fungemia is widespread among patients hospitalized with malignancies in the Paris area and is associated with a lower mortality than that of other *C. tropicalis* isolates. Despite a trend toward a decreased proportion over time, further studies are needed to assess this clone's geographic and temporal distribution. Analysis of the 2 PMMs described in this study, coupled with determination of nucleotide at position 529 in the *URA3* gene, should provide reliable means to track this clone.

Ms Desnos-Ollivier is an engineer at the National Reference Center of Mycoses and Antifungal at the Pasteur Institute in Paris, France. Her research interests include studying genotyping, sensibility to antifungal agents, and physiology of the yeasts responsible for human fongemia and underlining the relationship between genomic and phenotypic data.

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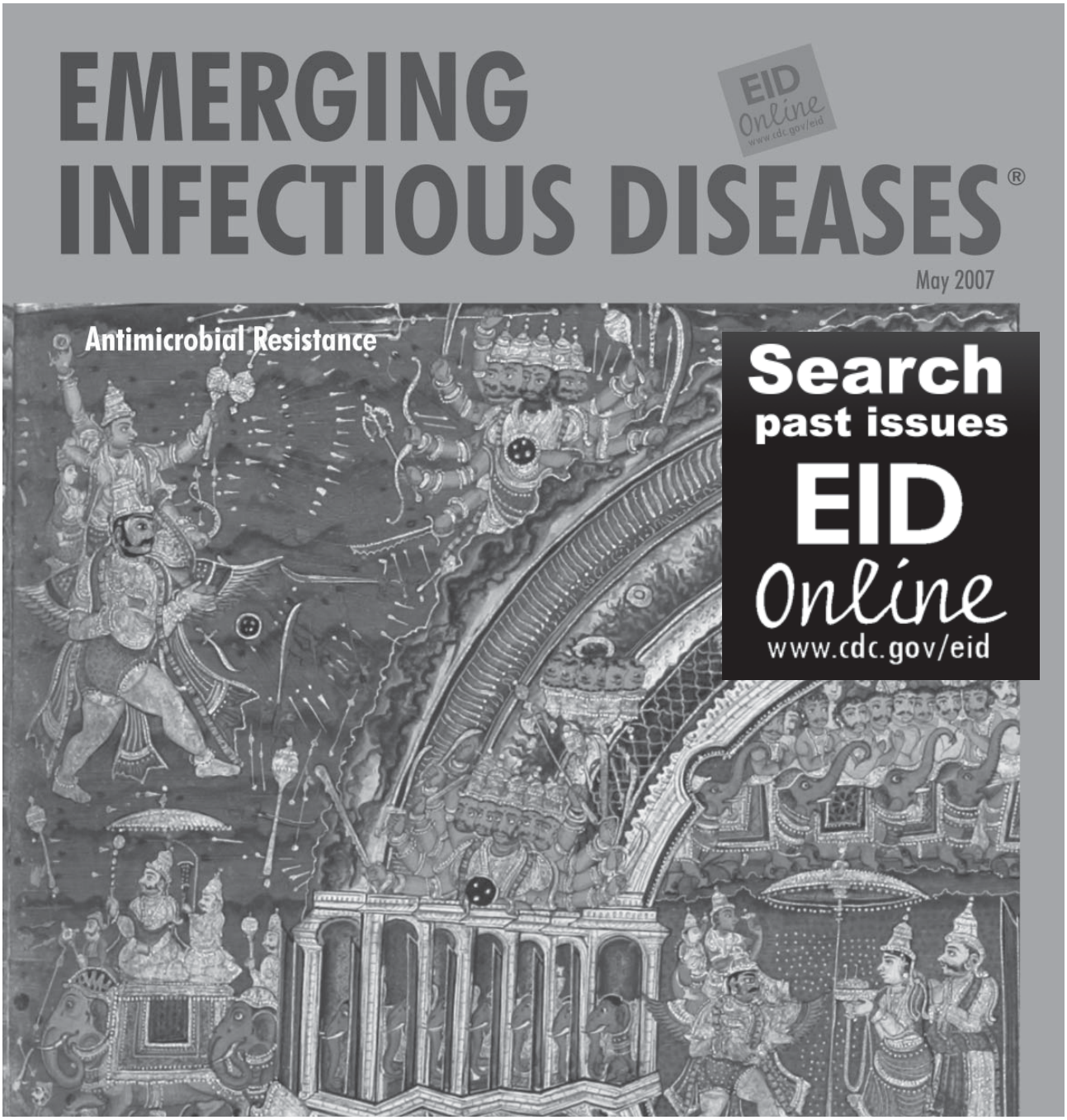

Emericella quadrilineata as Cause of Invasive Aspergillosis

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We noted a cluster of 4 cases of infection or colonization by *Emericella* spp., identified by sequence-based analysis as *E. quadrilineata*. Sequence-based analysis of an international collection of 33 *Emericella* isolates identified 12 as *E. nidulans*, all 12 of which had previously been identified by morphologic methods as *E. nidulans*. For 12 isolates classified as *E. quadrilineata*, only 6 had been previously identified accordingly. *E. nidulans* was less susceptible than *E. quadrilineata* to amphotericin B (median MICs 2.5 and 0.5 mg/L, respectively, p<0.05); *E. quadrilineata* was less susceptible than *E. nidulans* to caspofungin (median MICs, 1.83 and 0.32 mg/L, respectively, p<0.05). These data indicate that sequence-based identification is more accurate than morphologic examination for identifying *Emericella* spp. and that correct species demarcation and in vitro susceptibility testing may affect patient management.

The genus *Aspergillus* includes >250 species; ≈20 have been reported to cause opportunistic infections in humans. The most important human pathogens in this genus are *A. fumigatus, A. flavus, A. niger, A. terreus,* and *Emericella nidulans* (anamorph: *A. nidulans*) (*1*). *A. fumigatus* is the most common cause of invasive aspergillosis, a condition associated with substantial severity and mortality rates (*2*). Invasive infections caused by *E. nidulans* are uncommon in animals and humans (*3*–*5*); in humans they appear to occur predominantly in patients who have chronic granulomatous disease (CGD), a rare disorder of phagocytes in which the absence of both superoxide and hydrogen peroxide production in phagocytes predisposes patients to bacterial and fungal infections. Invasive *E. nidulans* infections in this patient group are associated with higher mortality rates than those caused by *A. fumigatus* (*6*,*7*). The most common site of infection is the lungs; other manifestations are subcutaneous abscesses or liver abscesses, suppurative adenitis, osteomyelitis, fungemia, cellulitis, and meningitis (*7*,*8*). Within the genus *Emericella*, other species have only rarely been identified as agents of human or animal infections.

The identification of *E. nidulans* in clinical microbiology laboratories is commonly based on the characteristic microscopic morphology, the production of hülle cells, or the production of ascospores. *A. fumigatus* is identified by its heat tolerance; other species fail to grow when incubated at high temperature, typically 48°C.

We recently noted a cluster of infection or colonization by *E. quadrilineata*, a species closely related to *E. nidulans*. Within a 3-month period, 4 cases were identified at the Radboud University Nijmegen Medical Center. No apparent epidemiologic link between the cases was found because each patient was cared for in a different ward, and 2 patients with invasive aspergillosis were admitted directly from home. One of the latter 2 patients was a 10-year-old boy with X-linked CGD and a probable diagnosis of invasive pulmonary aspergillosis (*9*); the other patient was a 60-year-old man who had chronic lymphocytic leukemia and in whom cerebral aspergillosis later developed and was confirmed by biopsy. From the other 2 patients, who had no signs and symptoms of invasive fungal disease, *E. quadrilineata* was cultured from respiratory specimens. No laboratory contamination was evident during the period in which the cluster occurred. No subsequent cases were identified. Morphologic species identification was difficult, and we had to rely on sequence-based identification, which prompted this investigation of the role of *E. quadrilineata* as a causative agent of invasive aspergillosis.

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Methods

Data and Strain Collection

We searched the PubMed literature for cases of infections caused by *E. quadrilineata* (anamorph: *Aspergillus tetrazonus*) or *E. nidulans;* search terms were *Emericella*, *quadrilineata, tetrazonus, nidulans*, and aspergillosis. For those articles that described infections by *E. quadrilineata* or *E. nidulans,* we asked the authors to send us their isolates for sequence analysis. We also approached colleagues who care for patients with CGD or might otherwise have a collection of *E. nidulans* isolates. We also searched our department's fungal culture collection for *E. nidulans* isolates. It is our policy to store all *Aspergillus* isolates cultured from clinical specimens sent to our laboratory, regardless of the clinical relevance of the isolate. Finally, we added *E. nidulans* and *E. quadrilineata* isolates deposited in the culture collection of the Centraal Bureau voor Schimmelcultures ([CBS], Utrecht, the Netherlands). The final collection totaled 33 *Emericella* isolates, with 11 isolates from the CBS culture collection (type strains *E. quadrilineata, E. nidulans, E. nidulans* var. *echinulata*) and 1 isolate from the National Collection of Pathogenic Fungi. Ten isolates were from our own culture collection (including the 4 encountered in the cluster of cases), and 11 isolates were from 5 other medical centers; some of these isolates had been cultured as causes of infection and previously reported (online Appendix Table, available from www.cdc.gov/EID/content/14/4/566-appT.htm) (*8*,*9*–*12*). Seven isolates had been cultured from patients with confirmed invasive aspergillosis, and 2 were from patients with probable cases (online Appendix Table).

Morphologic Identification

Aspergillus isolates are routinely identified by their macroscopic colony morphology and the microscopic morphology of their anamorphic features. When teleomorph features were also used to identify an isolate, teleomorph nomenclature, such as *Emericella* spp., was used to report the strain. In addition, the isolates were incubated at 48ºC, which precludes the growth of most *Aspergillus* spp. except *A. fumigatus*.

Sequence-based Identification

Sequence-based identification in the routine clinical microbiology laboratory was carried out by sequencing of parts of the internal transcribed spacer (ITS) 1 and 2 regions. Total DNA of the *Emericella* cultures was extracted by using the MagNa Pure Total NA isolation kit (Roche Diagnostics Nederland BV, Almere, the Netherlands). Then the ITS 1 and 2 sequence was amplified by PCR with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described (*13*). After purification, the PCR product was sequenced with the BigDye Terminator v3 kit (Applied Biosystems, Foster City, CA, USA).

In addition to the above-mentioned sequenced-based identification, parts of the β-tubulin and calmodulin genes were sequenced. The *Emericella* cultures were cultivated in 2 mL malt peptone broth by using 10% (vol/vol) of malt extract (Oxoid, Basingstoke, UK) and 0.1% (wt/vol) bacto peptone (Difco, Becton Dickinson, Le Pont de Claix, France). The cultures were incubated at 25°C for 7 days. DNA was extracted from the cells by using the Masterpure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. Amplification of part of the β-tubulin gene was performed by using the primers Bt2a and Bt2b (*14*,*15*). Amplifications of the partial calmodulin gene were set up as described (*16*). Sequence analysis was performed with the BigDye Terminator Cycle Sequencing Ready Reaction Kit for both strands. Sequences were aligned by using ClustalX software (*17*) and were improved manually.

Evolutionary distances between the sequences were calculated by using the Kimura formula (*18*) and DNADIST program of the PHYLIP program package (*19*). Phylogenetic trees were prepared by using the neighbor-joining method (*20*) and the NEIGHBOR program of the PHYLIP package. Bootstrap values were calculated from 1,000 replications of the bootstrap procedure by using programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE of the PHYLIP package (*19*,*21*). For parsimony analysis, PAUP* version 4.0 software was used (*22*). *E. heterothallica* was used as an outgroup in these experiments. The unique β-tubulin and calmodulin sequences were deposited in the GenBank nucleotide sequence database under accession nos. EF591677–EF591702.

Antifungal-Drug Susceptibility Testing

Antifungal-drug susceptibility testing of *Emericella* isolates was performed by using a microbroth dilution assay, as described by the Clinical Laboratory Standards Institute (M38-A) for amphotericin B (Bristol-Myers Squibb, Woerden, the Netherlands), itraconazole (Janssen-Cilag, Beerse, Belgium), voriconazole (Pfizer, Capelle aan den IJssel, the Netherlands), posaconazole (Schering-Plough, Maarsen, the Netherlands), caspofungin (MSD, Haarlem, the Netherlands), and terbinafine (Novartis, Arnhem, the Netherlands) (*23*). MICs were determined for all drugs except caspofungin, for which a microscopic endpoint was used (minimum effective concentration) (*24*). All in vitro susceptibility testing was performed in duplicate.

Statistical Analysis

After being transformed logarithmically, MIC dilutions were compared by using the Mann-Whitney U test.

Data on growth at different antifungal drug concentrations were normalized by setting the corrected optical density of the growth control at 0% and the lowest optical density at 100%. Growth characteristics were analyzed by nonlinear regression analysis that used a 4-parameter logistic model and created a sigmoidal curve. Test runs determined deviation of the model, and goodness-of-fit was tested by determining r^2 values. In addition to comparing MICs, we determined the drug concentration at which growth was 50% that of the control (50% maximal effective concentration [EC_{50}]) and calculated and fitted the slope of the curve (GraphPad Prism, San Diego, CA, USA). For all drugs except caspofungin, the EC_{50} values and slopes were compared for *E. nidulans* and *E. quadrilineata*.

Results

Species Identification

The 4 isolates from the cluster of cases grew on Sabouraud-dextrose agar as velvety, brownish-green colonies with a purplish reverse side. Conidiophores were light brown with hemispherical vesicles bearing metulae and biseriate philalides on the upper half. Conidia were spherical, smooth walled, subhyaline, finely roughened, and 3–4 μm in diameter. After ≈3 weeks of incubation, purple ascocarps formed, surrounded by characteristic hülle cells. Asci were spherical, 8 spored, 10–13 μm in diameter, and evanescent. The ascospores were reddish purple, lenticular, 5–6 × 3–4 μm, and smooth. The morphologic features were consistent with *E. nidulans.* As part of the diagnostic process, the isolates were incubated at 48ºC, and all isolates showed some growth, which was considered inconsistent with *E. nidulans*. Because of this discrepancy, sequence-based identification was performed. However, all 33 isolates from the subgenus *Nidulantes* section that were analyzed in this study grew at 48°C, which indicates that incubation at this temperature does not fully distinguish between this section and *A. fumigatus*.

Sequence-based Analysis

The ITS sequence analysis of the 4 isolates was consistent with that of *E. quadrilineata*, although there were only 1 or 2 mismatches with the base-pair sequence of *E. nidulans*. The morphologic features of *E. nidulans* and *E. quadrilineata* are very similar; only the microscopic shape of the ascospores shows subtle differences. Ascospores of *E. nidulans* have 2 longitudinal crests, as opposed to *E. quadrilineata,* which has 4 short equatorial crests. The resolution of the ITS region was considered too low to unambiguously differentiate between *E. nidulans* and *E. quadrilineata*, and further sequence-based identification was performed at CBS by using partial β-tubulin and calmodulin sequence data.

During analysis of part of partial β-tubulin gene sequences, we analyzed 367 bases of all 33 isolates. Among the polymorphic sites, 23 were phylogenetically informative. The neighbor-joining tree (Figure 1) based on partial β-tubulin gene sequences had the same topologic features as 1 of the 2 maximum-parsimony trees constructed by the PAUP program (length 94 steps, consistency index 0.9787, retention index 0.9762). The calmodulin dataset included 489 bases, with 50 parsimony informative sites. The topologic features of the neighbor-joining tree (Figure 2) and 1 of the 2 most parsimonious trees were the same (tree length 162, consistency index 0.9691, retention index 0.9854). Molecular data indicated that 12 of 33 isolates could be classified as *E. nidulans*, all of which had previously been identified as *E. nidulans* by microscopic examination of morphologic characteristics or other methods. For the 12 isolates classified as *E. quadrilineata*, only 6 had previously been identified accordingly. These 6 isolates included the 4 in our cluster, 1 from the CBS culture collection, and 1 previously reported as the cause of onychomycosis (*12*). The remaining 6 isolates had been previously identified as *E. nidulans* (online Appendix Table). Of these, 1 belonged to the CBS culture collection, 1 was reported as the cause of cerebral aspergillosis (*11*), and 2 were from patients with CGD and confirmed invasive aspergillosis.

A total of 4 isolates were classified as *E. rugulosa*, 1 of which had been previously reported as *E. nidulans* (*10*). A total of 4 isolates were identified as *E. nidulans* var. *echinulata*, 2 of which had caused invasive aspergillosis in patients with CGD and had been presumptively identi-

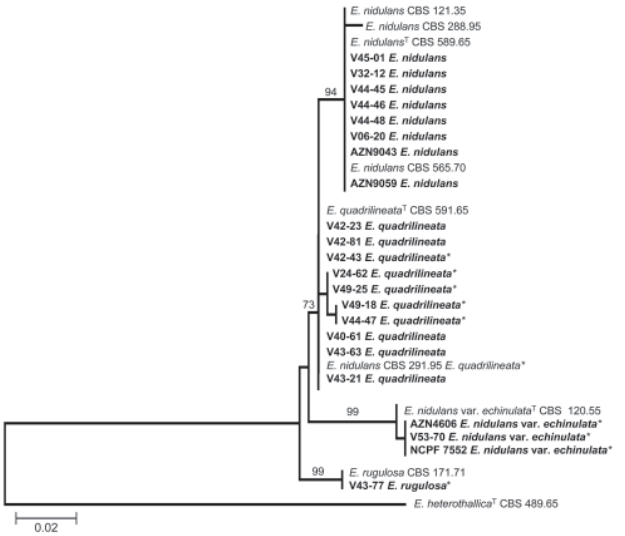

Figure 1. Neighbor-joining tree based on β-tubulin sequence data of the *Emericella* isolates examined. Clinical isolates are set in **boldface.** Numbers above branches are bootstrap values. Only values >70% are indicated. ^{T} indicates the type strain; * indicates the isolates that had been misidentified by morphologic identification as *E. nidulans*. Scale bar represents genetic distance calculated by the Kimura 2-parameter model (*18*).

fied as *E. nidulans*. Scanning electron microscopy of the ascospores of some isolates supported their species assignment (JEOL 5600LV scanning electron microscope [JEOL, Tokyo, Japan] equipped with an Oxford CT1500 Cryostation [Oxford Instruments, Oxford, UK]) (Figure 3) (*25*).

In Vitro Susceptibility

The in vitro activity of antifungal agents against the *Emericella* isolates is shown in the online Appendix Table. Overall, terbinafine was the most active drug in vitro, followed by posaconazole, which was the most active azole. For statistical comparisons, we used 12 *E. nidulans* and 12 *E. quadrilineata* isolates. By comparing MICs, statistically significant differences in drug activity were found for amphotericin B, voriconazole, and posaconazole (Table). These differences were also found when EC_{50} values and slopes were compared for both species (data not shown). Comparison of minimum effective concentrations showed caspofungin to be significantly more active against the *E. nidulans* isolates ($p<0.05$). Although only 4 *E. nidulans* var. *echinulata* isolates were analyzed, the susceptibility profile of these isolates was similar to that of *E. quadrilineata* (and not of *E. nidulans*); for amphotericin B, MICs were low, and for caspofungin, MICs were high.

Literature Review

Three cases of infection due to *E. quadrilineata* have been documented. One patient was a 60-year-old man from northern India, who had a fingernail infection that affected all 5 nails of 1 hand. The strain was repeatedly cultured from 1 nail, and septate hyphal elements were seen in a portion of an excised nail. The patient was treated with itraconazole, but the response could not be evaluated (*12*). Invasive aspergillosis caused by *E. quadrilineata* has been described for 2 patients, both of whom had sinusitis. One of these, a 28-year-old woman, had acute nonlymphoblastic leukemia and had undergone allogeneic bone marrow transplantation. She developed sinusitis with orbital involvement 2 months after transplantation. The diagnosis was confirmed by biopsy, and the patient was successfully treated with a combination of surgical debridement, granulocyte transfusions, and intravenous amphotericin B–cholesterol sulfate colloidal dispersion (*26*). The other patient was a 28-year-old man who had received an allogeneic bone marrow transplant for acute myeloid leukemia. Left orbital swelling, facial pain, and nasal congestion developed 68 days after transplantation. *E. quadrilineata* was cultured from a biopsy specimen; the patient was successfully treated with external ethmoidectomy, granulocyte transfusions, and topical and systemic therapy with a lipid formulation of amphotericin B. The fungal infection resolved (*27*).

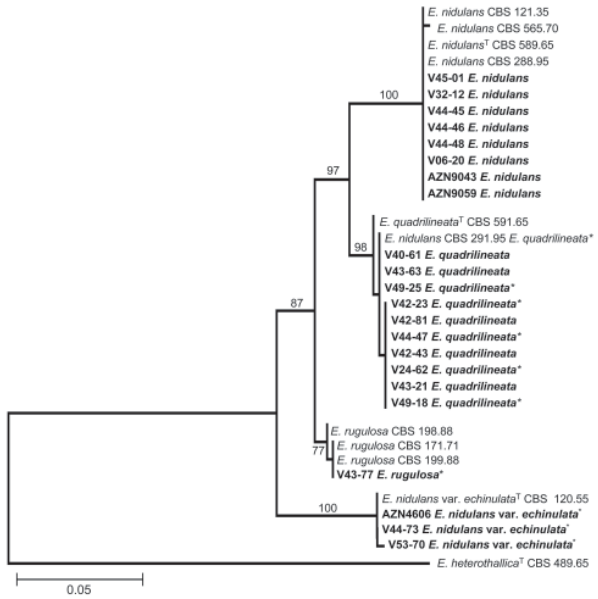

Figure 2. Neighbor-joining tree based on calmodulin sequence data of *Emericella* isolates examined. Clinical isolates are set in **boldface**. Numbers above branches are bootstrap values. Only values >70% are indicated. ^{T} indicates the type strain; * indicates the isolates that had been misidentified by morphologic identification as *E. nidulans*. Scale bar represents genetic distance calculated by the Kimura 2-parameter model (18).

Discussion

Until this report, 2 cases of invasive aspergillosis caused by *E. quadrilineata* had been described; each case had been reported as sinusitis in patients who had undergone bone marrow transplantation for hematologic malignancy. We add 1 case of central nervous system aspergillosis and 3 cases of invasive pulmonary aspergillosis in patients with CGD. The 3 cases may not be surprising because *E. quadrilineata* is very closely related to *E. nidulans*, a fungus known to cause infections in humans (*3*,*5*,*28–30*), primarily in patients with CGD (*6*,*7*). *Emericella* spp. other than *E. nidulans* are less frequently reported as causative agents of infectious disease. In addition to the above-mentioned human infections, *E. quadrilineata* has been identified as a causative agent of mycosis in animals (*31*). We have found only 1 report each of *E. rugulosa* (*4*) and *E. nidulans* var. *echinulata* (*32*) as the cause of human or animal infections.

Discriminating *E. nidulans* and *E. quadrilineata* by morphologic characteristics is virtually impossible. Only the ascospore ornamentation differs, and the subtle differences cannot be seen by using light microscopy. The most frequently used technique for their unambiguous identification is scanning electron microscopic examination of the lining of the ascospores (*25*). However, fruiting bodies are usually formed after a rather long incubation period (1–2 weeks). And although *E. nidulans* was found to form cleistothecia in the human body (*33*), clinical isolates often lose their ability to form sexual reproductive structures and

Table. Antifungal activities against *Emericella nidulans* and *E. quadrilineata*

Drug	MIC, mean		Significance*
	E. nidulans (n = 12)	*E. quadrilineata* (n = 12)	
Amphotericin B	2.5	0.5	p<0.05
Itraconazole	0.07	0.13	NS
Voriconazole	0.26	0.39	p<0.05
Posaconazole	0.25	0.22	p<0.05
Caspofungin†	0.32	1.83	p<0.05
Terbinafine	0.01	0.009	NS

*NS, not significant.
†Mean minimum effective concentration was compared.

ascospores (*34*,*35*). Given these difficulties, we anticipate that reliance on phenotypic characteristics alone would cause misidentification of *E. nidulans,* rather than correct identification of *E. quadrilineata,* as the cause of invasive aspergillosis. The sequence-based analysis showed that this was indeed the situation; 50% of the *E. quadrilineata* isolates had previously been identified as *E. nidulans*. Among these was a case of cerebral aspergillosis, the second case observed in our cluster of cases. Despite the close morphologic and genetic relatedness between *E. nidulans* and *E. quadrilineata*, the activity of antifungal agents differed significantly, which supports the conclusion that biological differences exist between these species. The triazoles were active in vitro; posaconazole showed the greatest activity, which is also observed for most *Aspergillus* spp. Although significant differences were found for activity of voriconazole and posaconazole, these differences appear to be not clinically relevant, given the small differences in MIC values (Table). However, for amphotericin B and caspofungin, the observed differences in activity may be clinically important. Amphotericin B was less active against *E. nidulans* than against *E. quadrilineata*. In vitro resistance of *E. nidulans* against amphotericin B has been recognized (*36*), although the testing method has been shown to substantially affect the activity found (*37*). However, lack of activity of amphotericin B has also been reported in experimental models of infection and in cases reported in the literature (*8*,*38*). Caspofungin was less active against *E. quadrilineata* than against *E. nidulans*. Caspofungin was shown to be effective against *E. nidulans* in a murine model of systemic infection (*38*), but no data are available for *E. quadrilineata.* Although the allylamine terbinafine is not used for treatment of patients with invasive aspergillosis, the drug is highly active against both *E. nidulans* and *E. quadrilineata*. The isolates were inhibited at concentrations as low as 0.015 mg/L.

Identification of molds primarily relies on morphologic criteria such as the macroscopic colony morphology and the microscopic morphology of the conidia and the structures bearing the conidia. Morphologic identification underestimates differences among species and among members of the same species. This was recently shown for the section *Fumigati*, in which *A. lentulus* and *A. udagawae* were among isolates phenotypically identified as *A. fumigatus* (*34*,*35*). We made a similar observation when 10 of 33 *Emericella* isolates were found to be misidentified. Correct species demarcation is important from a taxonomic viewpoint but can also have clinical relevance. Within the *Aspergillus* section of *Fumigati,* the newly identified species *A. lentulus* was shown to be more resistant than *A. fumigatus* to antifungal drugs (*35*). Therefore, correct species identification will affect the choice of antifungal therapy. Differences in drug activity were also apparent in the *Emericella* spp. we examined. Another important reason for correct species identification is the detection of outbreaks of infection, which could warrant interventions to prevent invasive fungal infection in immunocompromised persons or lead to epidemiologic surveys to identify sources of spread of fungal spores. However, the resolution of sequencing of the ITS region is too low to reliably dif-

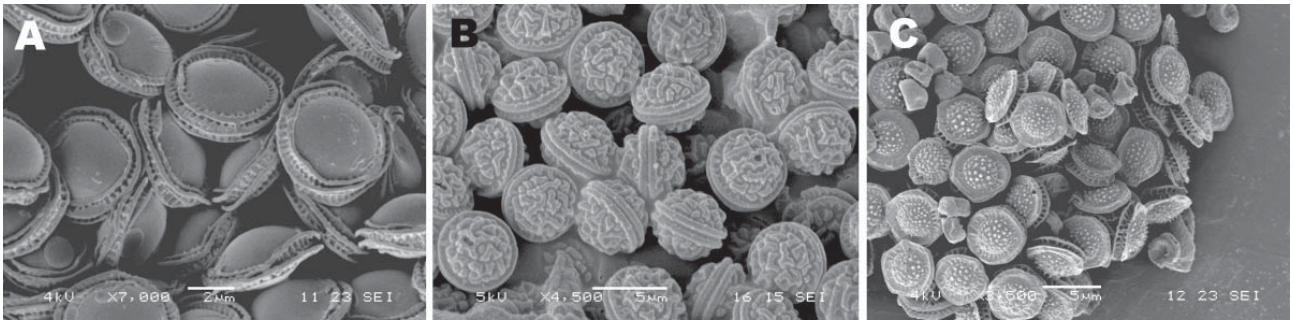

Figure 3. Scanning electron microscopic images of ascospores of some *Emericella* isolates. A) *E. quadrilineata* V43-63; B) *E. rugulosa* V43-77; C) *E. nidulans* var. *echinulata* 4606. Scale bars represent 5 μm.

ferentiate between *E. nidulans* and *E. quadrilineata;* therefore, in vitro susceptibility testing might be appropriate in those laboratories that do not have access to sequencing of β-tubulin and calmodulin genes.

Molecular techniques in addition to morphologic identification have identified a role of *E. quadrilineata* as an opportunistic fungal pathogen, especially in patients with CGD and in those with hematologic malignancy. These molecular techniques will help identify and discriminate more accurately within the current fungal species and will give more insight into the pathogenesis of fungal infection.

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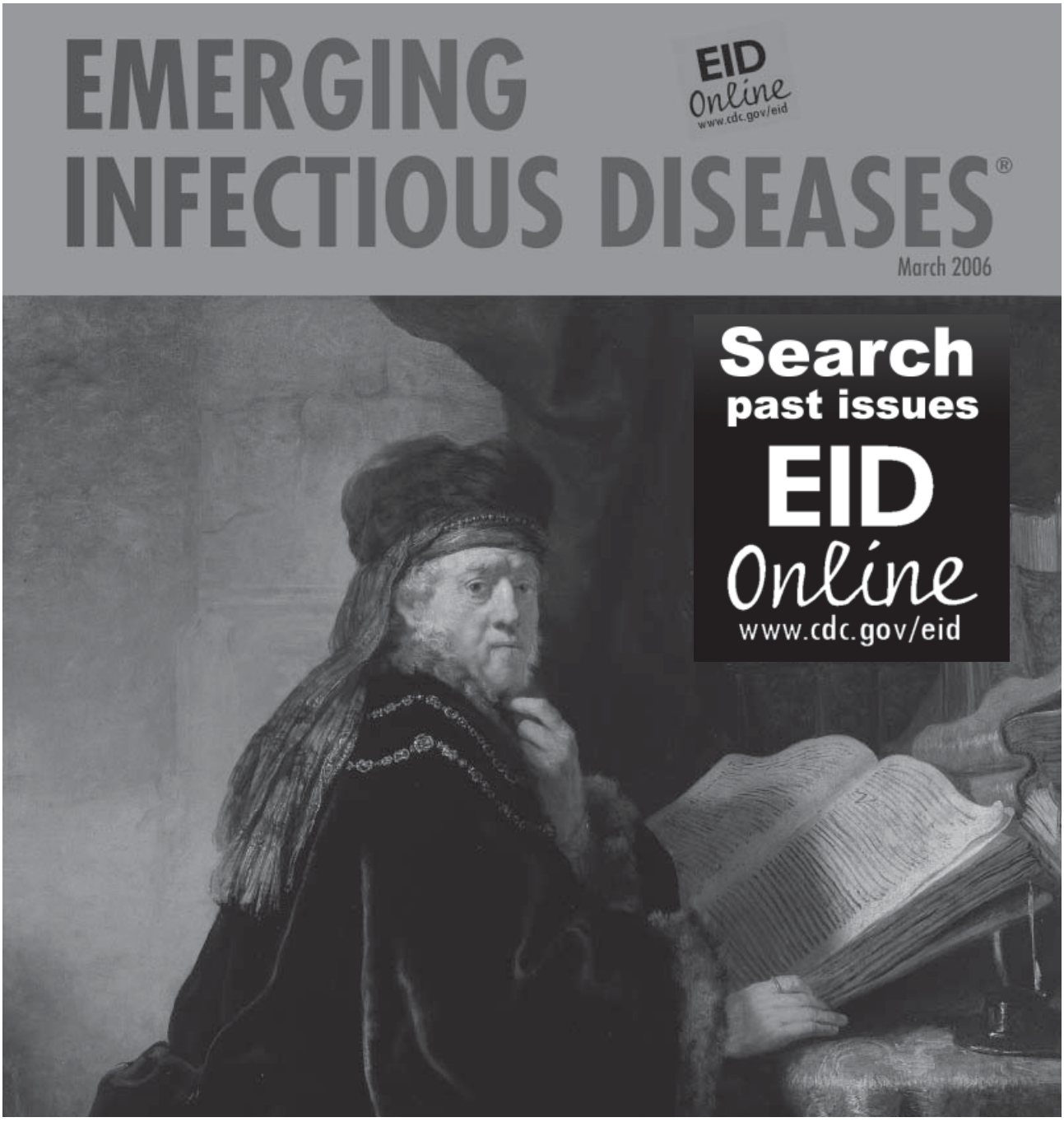

Control Measures Used during Lymphogranuloma Venereum Outbreak, Europe

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To assess the response to the reemergence of lymphogranuloma venereum, we conducted a cross-sectional survey by administering a structured questionnaire to representatives from 26 European countries. Responses were received from 18 countries. The ability to respond quickly and the measures used for outbreak detection and control varied. Evidence-based criteria were not consistently used to develop recommendations. We did not develop criteria to determine the effectiveness of the recommendations. The degree of preparedness for an unexpected outbreak, as well as the ability of countries to respond quickly to alerts, varied, which indicates weaknesses in the ability to control an outbreak. More guidance is needed to implement and evaluate control measures used during international outbreaks.

Responding effectively to international communicable disease emergencies is a complex process that involves national and international cooperation. Efforts should be aimed at managing patient care and containing the disease by interrupting the chain of transmission (*1*,*2*). The severe acute respiratory syndrome outbreak has shown the need for being prepared and being able to deal with international emergencies in a consistent way; all countries need to be prepared and able to respond to an outbreak. Countries throughout Europe have developed preparedness plans to face a possible pandemic caused by a new influenza virus. But even with a well-acknowledged threat like an influenza pandemic, differences in preparedness between countries exist (*3*,*4*). The differences might be even greater when timely control measures are needed for outbreaks that remain unnoticed for a long time.

Systems for surveillance and outbreak management among European countries differ, as do their health policies and guidelines. We wondered whether these differences could lead to different outbreak control measures and therefore to differences in the effectiveness of these control measures. We studied the quality and timeliness of public health actions during the reemergence of lymphogranuloma venereum (LGV) among men who have sex with men (MSM) in Europe from January 2004 to February 2006. In January 2004, the European Surveillance of Sexually Transmitted Infections Network (ESSTI) issued an international alert. This action was considered a trigger for countries to identify possible cases; define, inform, and investigate the population at risk; and to implement control measures. The resurgence of LGV in Europe contained many features similar to an infectious disease emergency: it occurred unexpectedly; there was delay in the recognition of cases, which allowed the disease to spread within the risk group; and there was no preconceived outbreak control plan. Moreover, in many countries, LGV is not reportable and surveillance is voluntary.

Our study of the response to this LGV outbreak demonstrates the need for a unified response to new, unexpected, infectious diseases. We assessed the similarities and differences in how various countries managed the LGV outbreak to identify common practices and to formulate criteria for improving the response to international outbreaks.

Participants and Methods

A cross-sectional survey was conducted from October 2005 through February 2006 among the countries participating in ESSTI and in Switzerland. The ESSTI then

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consisted of 22 member states of the European Union plus Iceland, Norway, and Turkey. Scotland was included as an individual respondent and participated in the network as such. In collaboration with the ESSTI steering group, we developed a structured questionnaire and sent it to each country's representative (surveillance leads and reference microbiologists).

The items on the questionnaire were based on a framework derived from the literature about outbreak management (*1*,*3*–*5*). In addition, to assess the quality of the development and implementation of key recommendations for controlling the outbreak, we used parts of the international AGREE instrument (www.agreecollaboration.org) for appraising guidelines and guideline development programs.

The questionnaire was divided into 4 sections. The first section was dedicated to the alert and initial response to LGV and included 8 questions about actions taken after the ESSTI alert, risk assessment, and occurrence of cases. The second section included 8 questions about the development of outbreak control measures and gathered information about how evidence was collected and analyzed, how measures were formulated, when experts were consulted, and how recommendations were updated. The third section included 9 questions about the content of outbreak control measures (i.e., case identification, case definitions, laboratory confirmation, treatment, reporting, and interventions for health professionals and the groups at risk). The fourth section addressed implementing outbreak control measures (i.e., strategies, dissemination of information, targets for monitoring effectiveness, and additional resources).

Questionnaires were completed electronically or on paper, and data were analyzed by SPSS 12.0 (Chicago, IL, USA). LGV is a sexually transmitted infection (STI) caused by *Chlamydia trachomatis* serovars L1, L2, and L3. Contrary to infection with other serovars, infections with *C. trachomatis* L1–3 are not limited to the mucosa but rather are often invasive and can spread to the lymph nodes, which results in a more severe clinical outlook. In industrialized countries, cases are incidentally imported from tropical and subtropical areas where the disease is endemic (*6*). Public health measures are usually restricted to contact tracing and adequate management of sex partners in individual cases; outbreak management is not needed. By the end of 2003, 13 cases had been reported to the public health authorities in the Netherlands, followed by a substantial increase in cases in subsequent months. The cases were seen among MSM. Clinical signs were mostly gastrointestinal and included proctitis, purulent or mucous anal discharge, and constipation (*7*). In the early days of the outbreak, the potential for international spread was recognized because patients reported having had sexual contacts in other countries such as Belgium, the United Kingdom, and France (*8*).

To create awareness, a message was sent through the Early Warning and Reporting System of the European Union and through the ESSTI. Since then, LGV cases have been identified in several European countries, the United States (*9*), and Canada (*10*). Most patients were HIV positive (*11*), and some were hepatitis C positive (*12*).

Results

The questionnaire was sent to 26 countries; 11 of these countries had reported outbreaks of LGV in the past. Completed questionnaires were received from 18 countries (Austria, Belgium, Denmark, Finland, France, Germany, Ireland, Italy, the Netherlands, Norway, Portugal, Scotland, Slovenia, Spain, Sweden, Switzerland, United Kingdom, and Turkey). Of the 18 questionnaires, 12 were completed by medical doctors, 4 by medical epidemiologists, and 2 by researchers/microbiologists. In 5 countries (Belgium, Ireland, Portugal, Slovenia, and Sweden), the questionnaire was filled in by 2 or more experts from different areas of expertise. The 8 countries that did not respond to either the questionnaire or the electronic reminders (Slovak Republic, Poland, Malta, Latvia, Iceland, Cyprus, Estonia, and Greece) were excluded from the analysis. A complete overview of the activities reported for controlling LGV and their development and implementation is given in the Tables 1 and 2.

Initial Alert and Response

After the ESSTI alert in January 2004, timely national alert and response systems were set up by 11 of the 18 responding countries. These systems included provisional control guidelines (9 countries), voluntary reporting (9 countries), and tools for disseminating information to health professionals (11 countries). Of the 11 countries who undertook early alert and response activities, 9 also reported cases. The main objectives of the alert were active case finding (11 countries), assessing the size and nature of the outbreak (10 countries), and providing appropriate clinical care (9 countries). In 4 countries, the initial alert and response were undertaken by professionals from the STI surveillance system in collaboration with specialists in outbreak control. In the other 7 countries, only surveillance specialists were involved.

Development of Outbreak Control Measures

Five countries had a national outbreak management team or advisory committee that provided scientific advice on surveillance and outbreak management. The multidisciplinary outbreak management teams always included epidemiologists and microbiologists; less frequently included were molecular biologists, dermatovenereologists, genitourinary specialists, and communicable disease control specialists. In 1 country, communication experts and social

scientists also participated in the outbreak management teams. No general practitioners, nurses, patients, or policymakers were involved in outbreak management teams. Of the 18 countries, control measures were aimed primarily at identifying new cases (8 countries) and promoting awareness among the risk group (10 countries) and STI clinics (11 countries). A risk assessment was performed by 8 countries.

When developing recommendations for outbreak control, criteria varied with the 18 countries (Tables 1 and 2). Evidence was systematically collected by literature (11 countries) and electronic database searches (10 countries). Informal consensus procedures were mostly used to formulate recommendations (10 countries) based on experience-based analysis of evidence (8 countries). Procedures for updating control measures were available in 11 countries.

Table 1. Control measures used by 18 European countries during an LGV outbreak*

Category	All countries (n = 18)	Countries with cases (n = 11)
Initial alert and response		
Alert and response issued	11	9
Enhanced surveillance	8	7
Voluntary reporting	9	7
Provisional guidelines	9	7
Information dissemination	11	9
Educational activities	6	6
Development of control measures		
Outbreak management team (advisory team)	5	5
Initial risk assessment performed	8	7
Methods to collect evidence		
Hand search literature	11	9
Search electronic databases	10	7
Search patient entry data	7	6
Search unpublished data	4	4
Methods to analyze evidence		
Decision analysis	5	3
Meta-analysis	0	0
Nonsystematic review	5	4
Systematic review	4	4
Experience based	8	7
Methods to formulate measures		
Subjective review	8	6
Informal expert consensus	10	8
Formal expert consensus	4	4
Evidence based	0	0
Procedure for updating key recommendations	11	4
National LGV guideline	4	3
Formal authorization process of the guideline	2	2
Content of control measures		
Active case finding	9	7
Contact tracing	7	6
Partner notification	5	4
Screening risk group	5	3
Activities targeting risk groups	10	8
Alerting general public	1	1
Alerting general practitioners	8	5
Alerting STI clinics	11	9
Alerting public health physicians	9	9
Alerting microbiologists	9	6
Alerting hospitals	5	3
Alerting GUM and gastroenterologists	8	6
Alerting HIV specialists	3	3
LGV notifiable†	5	4
National case-register for LGV†	9	7

*Data gathered through a survey. LGV, lymphogranuloma venereum; STI, sexually transmitted infection; GUM, genitourinary specialists.
†Data were missing for 3 countries for this category.

Table 2. Implementation of control measures used by 18 European countries during an LGV outbreak*

Implementation measure	All countries (n = 18)	Countries with cases (n = 11)
Disseminating educational materials	9	8
Conferences for professionals	3	3
National bulletins	5	4
Outreach visits	0	0
Computer reminders	0	0
Changes in medical records systems	0	0
Changes in facilities and equipment	0	0
Additional finances	3	3
Strategy for media communication	6	5
Involvement of MSM society in dissemination of information	11	8
Targets to monitor effectiveness	0	0

*Data gathered through a survey. LGV, lymphogranuloma venereum; MSM, men who have sex with men.

A total of 4 countries developed national, multidisciplinary guidelines for LGV control, 2 of which issued authorization procedures for the guidelines.

Content of Outbreak Control Measures

Active case finding was initiated by 9 countries and contact tracing by 7. Five countries implemented both. Information activities for the group at risk were performed by 10 countries and 11 countries alerted their STI clinics. An overview of all the control measures is given in the Tables 1 and 2. A total of 11 respondents (Denmark, Germany, Norway, Sweden, Spain, United Kingdom, Scotland, Austria, the Netherlands, Ireland, and Belgium) used an identical case definition for confirmed cases: MSM or sexual contacts of patients with LGV, who had anorectal or inguinal syndrome and positive nucleic acid amplification tests (NAAT) for *Chlamydia trachomatis* genotype L1, L2, or L3. From these 8 countries, case definitions were also issued for probable and possible cases and differed widely according to laboratory criteria.

Laboratory diagnosis of *C. trachomatis* was performed by NAAT on the following samples: rectal swabs (12 countries), biopsy material from lesions (8 countries), urethral swabs (5 countries), and urine (2 countries). Genotyping to confirm the presence of serovars L1–L3 was also available from 11 countries. Supplementary testing of patients for concurrent STIs was recommended as follows: HIV (8 countries), syphilis (5 countries), hepatitis C (3 countries), hepatitis B (3 countries), and *Neisseria gonorrhoeae* (2 countries).

With respect to antimicrobial therapy, various regimens and different doses were used. For 9 countries doxycycline (100 mg 2×/day for 21 days) was the first choice of treatment. Alternatives mentioned were tetracycline (2 g/day), minocycline (300 mg loading dose followed by 200 mg 2×/day), and erythromycin (500 mg 4×/day). Clinical and laboratory follow-up of the patients was performed by 10 countries.

Implementation of Outbreak Control Measures

The control measures were implemented by disseminating educational materials in 9 countries, disseminating national bulletins in 5, and holding meetings and conferences for professionals in 3 countries. Most countries (11/18) had the risk group help disseminate information. Targets to monitor the effectiveness of recommendations were not formulated by any country.

Discussion

This outbreak of LGV had special features with high clinical and public health significance. First, recognition of cases was difficult due to the unusual clinical presentation that mimics inflammatory bowel disease. Second, the diagnosis of LGV involved invasive procedures for collecting samples and required NAAT, which were not licensed for rectal specimens. Furthermore, patients mostly belonged to sex networks of MSM in large cities with numerous anonymous partners from different countries (*13*) and where (international) contact tracing was difficult. In most European countries, LGV is not notifiable by law so cases are likely to be dealt with outside the public health domain. The potential of unnoticed further spread and the risk for simultaneous transmission of other infections, such as HIV and syphilis, increased the public health importance of this outbreak.

Differences were seen between countries with respect to ability to rapidly respond and implement measures that are needed to detect or to control a possible outbreak. Countries that reported cases of LGV were more likely to recommend control measures although measures were also needed to detect possible cases. To identify and diagnose cases, clinical specialists and public health physicians, as well as the risk group, must be aware of the outbreak, particularly for an LGV outbreak. LGV is a rare disease in Europe, and often healthcare workers are not aware of the clinical features of the disease.

Outbreak control measures require collaboration between persons in multiple specialties, such as specialists in surveillance, communicable disease control, health promotion, and physicians involved in the direct patient care (venereologists, genitourinary medicine specialists, gastroenterologists, microbiologists) that do not necessarily work together in other circumstances. In this outbreak, information from the surveillance systems was as important for health providers as for policymakers; this information had to lead to immediate recognition of a public health threat and direct action.

However, in the management of LGV patients, differences were seen between countries with respect to case definitions, laboratory testing, and antimicrobial drug treatment. With most patients belonging to international sex networks, uniform diagnostic procedures and treatment protocols would have been helpful for ensuring a uniform approach to outbreak control. Furthermore, control measures were impaired because in many countries LGV is not a notifiable disease; therefore, there is no legal basis for disclosing names of sexual contacts to facilitate contact tracing and prevent further spread. Contact tracing was made even more difficult because of the numerous anonymous sexual contacts in various European cities.

Criteria for evidence-based development of recommendations were not always consistently used to extract and analyze evidence for best practices during the LGV outbreak, which led to differences in outbreak management. Specific targets for monitoring the effectiveness of recommendations were not formulated by any country. One strong point was the acknowledgment by many countries of the importance of having the risk group, MSM, disseminate alerts and advocate awareness.

Until now, the reemergence of LGV has affected MSM in 11 European countries. The ESSTI alert prompted these countries to take action to identify cases early, improve the management of cases, and assess the size of the outbreak. Of the 18 respondents, 7 stated that they had not taken any action at this stage for various reasons: they did not receive the alert (Turkey, Slovenia) or they did not participate in the ESSTI (Switzerland). Coordination at the European level should encourage and monitor the response of all countries to alerts.

Our study has some limitations. We assumed that all countries that were participating in the ESSTI network in 2005 had also been informed about the LGV outbreak. Later, it became clear that the countries that had joined the European Union on May 1, 2004, did not receive the ESSTI alert. Because only 1 of these new European Union member countries completed the questionnaire, it was also impossible to assess how outbreak control measures were developed and implemented. Another limitation inherent to the method used was that not all key persons involved in the control of LGV were able to fill out the questionnaire. As the questionnaire was sent to the country representatives in the ESSTI, it is possible that not all relevant information was available on the control measures and activities that had taken place at regional or local levels. Furthermore, the quality of the outbreak management process and the development of outbreak measures could only be assessed indirectly on the basis of the answers to the questionnaire because only a few countries provided more detailed documents like guidelines or articles. The LGV outbreak is still ongoing in Europe, and since the completion of this study more countries may have undertaken measures to identify and treat cases and to prevent further transmission.

Our findings are helpful for understanding the responses to unexpected disease outbreaks. However, we do acknowledge that LGV is an STI (rather than a quick-spreading communicable, airborne disease) and therefore, affects a minority of sexually active citizens (MSM) in the country.

Communicable diseases differ from other health threats or crises because they spread from person to person. Therefore, problems are often not restricted to 1 country. Various specific interventions are therefore justified by the difference in local systems, cultures, and situations. However, the principles of outbreak response are general, and countries can learn from each other. This study shows that countries have varying degrees of ability to respond quickly to an unexpected outbreak; these findings expose weaknesses in the outbreak control capacity in Europe. Although important steps have been taken for improvement (*14*), the quality of LGV outbreak control in Europe could benefit from uniform approaches in controlling other infectious diseases with potential for international spread and from exchanging information between countries.

The challenge for the future will be to coordinate outbreak management in various countries for which continuity and coherence are essential. This study shows that coordination should at least aim to provide guidance as to when and how alerts should be implemented by various countries as well as to establish uniform case definitions and ensure the availability of optimal diagnostic facilities. We also show a lack of common strategies and that these should be developed with respect to treatment algorithms and contact tracing. Furthermore, quality systems following the whole chain of outbreak management (alert, outbreak control measures, implementation, and evaluation) are needed. These systems should be based on standard approaches to outbreak management followed by external review of implemented measures. More international collaboration is needed to improve response and to ensure high standards of practice in managing international outbreaks and threats caused by emerging or reemerging STIs.

Crisis situations increase the chance of overlooking essential steps in outbreak management because of time constraints, uncertainty, and the lack of substantial evidence in effective approaches to controlling new diseases. Furthermore, during outbreaks, key recommendations involve quick decision-making by professionals who often have no time for reevaluation. Although this need for quick decisions has been acknowledged for other threats like avian flu, SARS, or bioterrorism, little experience has been acquired with managing outbreaks of STIs. Our systematic approach could be helpful in preparing for or assessing the response to all kinds of public health emergencies.

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CME ACTIVITY

β-Herpesviruses in Febrile Children with Cancer

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Identify common infections associated with β–herpesviruses
- Specify β–herpesviruses isolated from children in the current study
- Describe clinical characteristics of β–herpesvirus infections in the current study
- List factors associated with higher rates of infection with human herpesvirus in the current study

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β-Herpesviruses in Febrile Children with Cancer

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We conducted a cross-sectional study of β-herpesviruses in febrile pediatric oncology patients (n = 30), with a reference group of febrile pediatric solid-organ transplant recipients (n = 9). One (3.3%) of 30 cancer patients and 3 (33%) of 9 organ recipients were PCR positive for cytomegalovirus. Four (13%) of 30 cancer patients and 3 (33%) of 9 transplant recipients had human herpesvirus 6B (HHV-6B) DNAemia, which was more common within 6 months of initiation of immune suppression (4 of 16 vs. 0 of 14 cancer patients; p = 0.050). HHV-6A and HHV-7 were not detected. No other cause was identified in children with HHV-6B or cytomegalovirus DNAemia. One HHV-6B–positive cancer patient had febrile disease with concomitant hepatitis. Other HHV-6B–positive children had mild "viral" illnesses, as did a child with primary cytomegalovirus infection. Cytomegalovirus and HHV-6B should be included in the differential diagnosis of febrile disease in children with cancer.

Much remains to be learned about the pathogenic role of β-herpesviruses (cytomegalovirus [CMV], human herpesvirus 6 variants A and B [HHV-6A and HHV-6B], and human herpesvirus 7 [HHV-7]) in immune-compromised children. Most persons are infected with CMV, HHV-6B, and HHV-7 during childhood; the age of acquisition and clinical spectrum of HHV-6A have not been defined. In immune-competent children, CMV is associated with heterophile-negative mononucleosis, HHV-6B with roseola infantum (exanthem subitum or sixth disease), and HHV-7 with a small percentage of clinically recognized cases of roseola. However, most primary infections with these viruses are either asymptomatic or involve a nonspecific mild illness that can include fever, malaise, and abnormal liver function or hepatosplenomegaly (*1*–*4*). After primary infection, these viruses establish life-long residency in the host, seldom causing disease unless the immune system is weakened, as occurs after treatment for solid-organ and stem cell transplantation. In these patients, each of the β-herpesviruses can reactivate, manifesting as febrile and sometimes life-threatening illness including pneumonitis, encephalitis, bone marrow suppression, graft-versus-host disease, and organ rejection (*5*–*7*). In addition to having independent pathologic effects, β-herpesviruses may have additive or synergistic effects, as well as interactions with other infectious agents (e.g., fungal infections) (*8*,*9*).

Immune suppression caused by cancer treatment has many forms, often as pulses of cytotoxic agents that kill rapidly dividing cells, including lymphocytes. The risk for infections in pediatric cancer patients is well recognized, and much effort has been devoted to identifying and treating bacterial and fungal infections associated with fever and neutropenia (*10*–*14*). This effort usually involves hospitalization for empiric administration of intravenous antimicrobial drugs, even though most bacterial blood cultures remain negative; 40%–70% of such febrile episodes have no identifiable source (*15*,*16*). Some viral infections, such as those with herpes simplex or varicella zoster viruses, are associated with disease that can be serious and even fatal in pediatric oncology patients (*17*,*18*). Most episodes of fever are unexplained and assumed to be viral in nature (*19*).

Little attention has been paid to the possible contribution of β-herpesviruses as a cause of febrile illness in children with cancer, despite recognition that these viruses cause disease after organ transplantation. In studies that preceded application of PCR, CMV detection was associated with fever and hepatitis in children with malignancy

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(*20*,*21*). HHV-6 seroprevalence is similar in pediatric cancer patients and controls (*22*,*23*), but virus has been detected less frequently in saliva of children with cancer than that of healthy controls (*24*). In children from the Czech Republic, Michalek et al. detected both primary and reactivated HHV-6 and CMV infections during cytotoxic chemotherapy by using serologic analysis and PCR (*23*,*25*). Some HHV-6 infections were associated with severe disease, including pneumonitis, bone marrow aplasia, and persisting fever.

Because of the biologic plausibility of β-herpesvirus involvement in febrile illness in pediatric cancer patients and the paucity of PCR-era literature in this area, we conducted a cross-sectional study of the activity of these viruses in pediatric cancer patients and other immune-compromised children. The purpose of this study was to determine whether there is sufficient viral activity in these populations to warrant in-depth study and clinical consideration.

Materials and Methods

Patients

The study was reviewed and approved by the Cleveland Clinic Institutional Review Board. Informed consent was obtained from a parent or guardian of each person <18 years of age, or directly from persons ≥18 years of age; assent was obtained from children 7–17 years of age. Patients were enrolled from August 2004 through April 2005. Enrolled children were receiving treatment for a malignancy or were receiving immunosuppressive therapy after solid-organ transplantation (SOT). Inclusion criteria were an age of newborn to 21 years and new onset of fever with an oral or rectal temperature ≥38°C or an axillary temperature ≥37.5°C. At enrollment, we collected a blood specimen and information on age, sex, underlying illness and diagnosis, acute symptoms accompanying fever, and details about immune suppression (chemotherapy regimen, radiation therapy, immune modulators, and steroids). Gastrointestinal symptoms were defined as either vomiting or diarrhea. Clinical laboratory data collected included complete blood counts, renal and liver function test results, bacterial blood cultures, as well as results of testing conducted for evaluation of fever, such as respiratory virus analysis, stool studies, chest radiographs, and computed tomography scans.

Specimens and Storage

Whole blood was collected in EDTA tubes and stored at 4°C until processed. A leukocyte pellet was collected from 1 mL of whole blood; 400 μL of plasma was preserved, 200 μL for PCR and 200 μL for serologic analysis. Pelleted leukocytes were added to MagnaPure LC lysis buffer (Roche Diagnostics, Indianapolis, IN, USA) and stored at –20°C.

Virus Culture

CMV culture was conducted with pelleted leukocytes by using conventional MRC-5 cell shell vial cultures (Diagnostic Hybrids, Athens, OH, USA). Immune fluorescence testing was conducted after 48 hours by using a monoclonal antibody directed against the CMV immediate early protein (Chemicon, Temecula, CA, USA). Shell vial cultures were obtained for the first 11 patients enrolled; all cultures were negative. Because shell vial culture uses a large number of leukocytes and we were studying a neutropenic population, this procedure was discontinued to preserve cells for more sensitive PCR analysis (*26*). Purified lymphocytes were cultured to detect HHV-6 or HHV-7 infections as described (*27*,*28*). Cytopathic effect was recognized as enlarged and refractile cells, with confirmatory testing by PCR.

Polymerase Chain Reaction

Template DNA was extracted by using the MagnaPure automated extraction method (Roche Diagnostics) and eluted into 50 μL of buffer. A total of 5 μL of eluted DNA was used in each PCR. The PCRs contained DNA extracted from the equivalent of ≈20 μL of plasma, leukocytes present in 100 μL of whole blood, and 20 μL of whole blood. For detection of CMV and HHV-6 variants A and B, the Artus CMV RG PCR ASR/Real Art/Qiagen RealArt CMV LC Assay and the RealArt/Qiagen HHV-6 A/B LC Assay (QIAGEN, Hamburg, Germany) were used. A co-amplified internal control was used to identify PCR inhibition. To differentiate between HHV-6 variants, the system uses the specific melting temperatures of the probes. Detection of HHV-7 was conducted by using previously described primers and hybridization probes (*29*). Real-time PCR was performed on a LightCycler system (Roche Diagnostics).

Serologic Analysis

CMV serologic analysis was performed by using the VIDAS CMV IgG Assay (bioMérieux, Durham, NC, USA) in an automated instrument, according to the manufacturer's protocol. Analyses for HHV-6 (using HHV-6A–infected cells) and HHV-7 were conducted by using conventional indirect immunofluorescence with commercial slides (Panbio Ltd, Columbia, MD, USA). Avidity of antibody to HHV-6 was tested as described by Ward et al. (*30*).

Statistical Analysis and Clinical Associations

The exact unconditional test was used to compare groups on viral activity (*31*). All tests were 2-tailed and performed at a significance level of 0.05. Analyses were performed by using StatXact PROCs version 6.3 (Cytel Software Corp., Cambridge, MA, USA) and SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Clinical associations with β-herpesvirus infection were based on symptoms

and concomitant laboratory findings in patients who had no other identifiable cause for their febrile illness.

Results

Patient Characteristics

Forty-one children were enrolled, from whom 39 evaluable specimens were available (Table 1). Thirty of the 39 children had a malignancy (our primary interest) and 9 were organ transplant recipients who were included for comparison. All cancer patients were receiving active therapy, except for 1 child who was included in the study because she had been receiving chemotherapy for 2.5 years, which ended 3 months before study enrollment. Several patients had symptoms in addition to fever, mostly upper respiratory or gastrointestinal. A source of fever was identified for 5 (17%) of 30 cancer patients and 2 (22%) of 9 organ transplant recipients. Two cancer patients had bacteremia (*Escherichia coli*, coagulase-negative staphylococci); no fungal infections were detected. Three cancer patients and 2 organ transplant recipients had respiratory viral infections diagnosed by symptoms and positive direct fluorescent antibody test results. Of the 39 children, levels of liver enzymes were obtained for 28 as part of routine care. Of these children, 6 had abnormal values; 2 of these 6 also had virus detected (HHV-6B in 1 patient and influenza A in the other). The other 4 patients with increased levels of liver enzymes had longstanding abnormalities; review of their records suggested that the abnormal results were caused by underlying disease in 1 liver transplant patient and chemotherapy in 3 cancer patients. Six patients have died since completion of our study, none from infectious causes.

Table 1. Characteristics of febrile immune-suppressed children*

Characteristic	Cancer (n = 30)	SOT (n = 9)
Age, y; mean, median, range	6.1, 5.0, 0.4–17	11.8, 13.3, 4.3–20.6
Sex		
F	14	7
M	16	2
Basis of immune suppression		
Cancer	30 (77%)	–
Solid tumor	20	–
Leukemia	9	–
Lymphoma	1	–
Solid-organ transplant	–	9 (23%)
Heart	–	5
Lung	–	2
Liver	–	2
Immune suppressants	29	9
Cytotoxic chemotherapy	28	–
Radiation	6	–
Chemotherapy and radiation	6	–
Immune modulators†	–	9
Steroids (past 6 months)	14	7
Signs and symptoms		
Ill appearance	7	1
Upper respiratory	17	1
Gastrointestinal	10	7
Headache	3	3
Rash	0	0
Seizure	0	0
Hepatitis	1	1
Blood products (past 6 months)‡	20	5
Laboratory parameters		
Neutropenic (<500 cells/μL)	14	2
Lymphopenic (<500 cells/μL)	13	4
Established source for fever		
Bacteremia	2	0
Positive respiratory DFA test result§	3	2

*SOT, solid-organ transplantation; –, not applicable; DFA, direct fluorescence antigen.
†Immune modulators included tacrolimus in 4, tacrolimus and azathioprine in 2, cyclosporine and mycophenolate mofetil in 2, sirolimus in 1, and tacrolimus, mycophenolate mofetil, antithymocyte globulin (rabbit), and corticosteroids in 1.
‡Blood products recorded included packed erythrocytes or platelets.
§Of the 5 patients with positive respiratory DFA test results, 2 were positive for parainfluenza 3, 2 for influenza A, and 1 for respiratory syncytial virus.

Laboratory Results

Of the 11 specimens tested in the shell vial assay, none was positive for CMV. CMV DNA was detected by PCR in lymphocytes of 1 (3.3%) of 30 cancer patients and 3 (33%) of 9 organ transplant recipients (Table 2).

Lymphocyte cultures for HHV-6 and HHV-7 were obtained for 34 of the 39 children. Of these, 15 cultures showed cytopathic effects similar to those commonly reported for HHV-6, but none of these were positive for HHV-6 by PCR. Cultured cells from 1 patient with leukemia (study participant 19) were positive for HHV-6B by PCR, in the absence of a cytopathic effect. Four (13%) of 30 cancer patients and 3 (33%) of 9 transplant recipients (p = 0.19) had HHV-6B DNAemia; HHV-6A was not detected. Viral loads ranged from 50 to 500,000 DNA copies/mL. Of 7 PCR-positive patients, 3 also showed positive results in peripheral blood mononuclear cells (PBMC) and whole blood specimens, 2 in only PBMC, and 1 only in a lymphocyte culture. Patient 40, a transplant recipient, had positive results in all specimens tested including PBMC, whole blood, and plasma, with the highest viral load (>5×10^5 genomes/mL) in PBMC. These results are consistent with either severe acute infection or the child having germline integrated HHV-6B (*32*,*33*). PCR positivity was more common in children whose blood was collected within 6 months of initiation of immune suppression compared with children sampled >6 months after initiation of immune suppression (cancer patients: 4 of 16 vs. 0 of 14 after 6 months, p = 0.050; transplant recipients: 2 of 3 vs. 1 of 6, p = 0.26) (Figure). Solid-tumor patients were less likely to be positive for HHV-6B than either leukemic (1 of 20 vs. 3 of 9,

Table 2. Detection of β-herpesviruses in cancer patients and solid-organ transplant recipients*

Characteristic	Cancer patients, no. positive/no. tested (%)		Transplant patients (n = 9), no. positive/no. tested (%)
	Leukemia (n = 9)	Solid tumor (n = 20)	
CMV PCR			
Whole blood	1/6 (17)	0/5	3/7 (43)
PBMC	1/7 (14)	0/16	2/8 (25)
Plasma	0/9	0/20	2/9 (22)
All tests	1/9 (11)	0/20 (0)	3/9 (33)
HHV-6 PCR			
Whole blood	2/6 (33)	0/5	2/7 (29)
PBMC	1/7 (14)	1/16 (6)	3/8 (38)
Plasma	0/9	0/19	1/9 (11)
Lymphocyte culture	1/8 (13)	0/16	0/9
All tests	3/9 (33)	1/20 (5)	3/9 (33)
HHV-7 PCR			
Whole blood	0/6	0/5	0/7
PBMC	0/7	0/16	0/8
Plasma	0/9	0/20	0/9
All tests	0/9	0/20	0/9

*Data are not shown for study participant 23, a patient with lymphoma who did not have β-herpesviruses detected in whole blood, PBMC, or plasma. CMV, cytomegalovirus; PBMC, peripheral blood mononuclear cells; HHV-6, human herpesvirus 6.

p = 0.046) or SOT patients (1 of 20 vs. 3 of 9, p = 0.046). Two of the HHV-6B-positive SOT patients had concurrent CMV DNAemia.

HHV-7 DNA was not detected in any patients. Lymphocyte cultures were not tested for HHV-7 by PCR because none of the plasma, whole blood, or leukocyte pellets were positive for the virus.

Possible Associations between β-Herpesvirus Activity and Disease

Patients who were positive for β-herpesviruses and those who were negative for these viruses did not differ in their average fever temperature (38.5°C vs. 38.6°C). In SOT recipients, the prevalence of HHV-6B and CMV and the spectrum of their associated illnesses were concordant with prior reports. Thus, we will focus on the cancer patients.

The only cancer patient with CMV DNAemia appeared to have primary CMV infection on the basis of negative results for immunoglobulin (Ig) G to CMV, negative bacterial blood culture, and age (online Appendix Table, available from www.cdc.gov/EID/content/14/4/579-appT.htm). This patient was a 5.4-year-old child (study participant 29) with acute lymphoblastic leukemia (ALL) in remission; he had upper respiratory symptoms and fever but otherwise appeared well. He was treated with an antimicrobial drug (ceftriaxone) on an outpatient basis, but his blood culture was negative for bacteria and no other source for his fever and illness was found. This child began cancer chemotherapy 36 months before the febrile event.

Four patients appeared to have reactivated HHV-6B infection on the basis of positive results for virus DNA, high-avidity IgG, and clinical symptoms. The first patient (study participant 19) was a 2-year-old boy with ALL in remission for the first time. In the second month of chemotherapy; he was hospitalized for fever and ill appearance, but he was not neutropenic or lymphopenic. He had marked hepatomegaly (≈10 cm below the right costal margin) and abnormal results in liver function tests. He was receiving fluconazole for oral thrush, and his liver enzyme levels were mildly increased before the febrile illness. However, at admission he had an aspartate aminotransferase (AST) level of 2,356 U/L and an alanine aminotransferase (ALT) level of 2,410 U/L. HHV-6 and HHV-7 IgM and IgG titers were obtained (Specialty Laboratories, Valencia, CA, USA) as part of his ALL treatment protocol. His HHV-6 IgM titer was negative (<40), and his IgG titer was positive (320) and high-avidity antibodies were detected. Results for HHV-7 IgM were negative, and those for IgG were weakly positive (titer 20). Other studies conducted by the primary medical team included tests for acute hepatitis and a CMV PCR; all results were negative. Four days later, his AST level was 84 U/L and his ALT level was 314 U/L. On follow up 7 days after admission, both values were near normal. The hepatitis was attributed to methotrexate, which was therefore withheld until the levels of liver enzymes returned to normal. On the basis of the positive culture for HHV-6B and hepatitis in the setting of febrile illness without other identified infections, we suspect that reactivated HHV-6B infection caused the fever and exacerbated the hepatitis.

A 5-year-old girl with relapsed acute myeloid leukemia (study participant 23) was intermittently febrile while hospitalized for 1 month during chemotherapy. She was treated with broad-spectrum antibacterial drugs and antifungal drugs. At study enrollment on day 12 of hospitalization, she had an absolute neutrophil count of 0 and an absolute lymphocyte count of 560 cells/μL. Daily blood cultures and sporadic respiratory viral studies did not re-

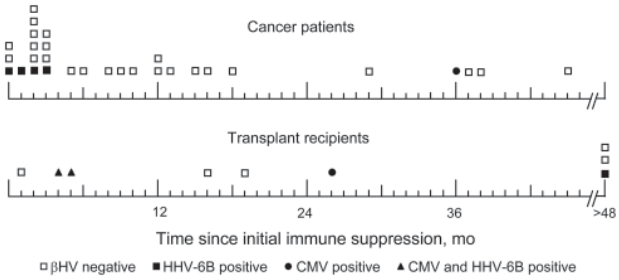

Figure. Temporal relationship between detection of β-herpesvirus (βHV) and onset of immune suppression. Time of collection of each study specimen is indicated relative to onset of immune suppression for cancer patients and solid-organ transplant recipients. HHV, human herpesvirus; CMV, cytomegalovirus.

veal a cause for her fever, but her whole blood and PBMC were positive for HHV-6B by PCR.

HHV-6B was detected in a 2-year-old girl with ALL in remission (study participant 25) and in a 22-month-old boy with stage 3 Wilms tumor (study participant 38). Both of these patients were lymphopenic and had nonspecific febrile illnesses; neither had other identified sources of fever.

We suspect primary CMV infection in 1 patient and reactivated HHV-6B infection in 4 cancer patients as likely causes of the febrile episodes. This conclusion is based on a combination of clinical data, positive PCR findings, and lack of other identified sources for fever.

Discussion

β-Herpesviruses are known pathogens in immunocompromised patients, but they have not been extensively studied in children with malignancies. In this study of children who had fever, signs of infection, and had samples taken at 1 time point, we detected β-herpesviruses in 5 of 25 cancer patients who had no other source of fever identified: 4 had HHV-6B DNA and 1 had CMV DNA detected by PCR. β-Herpesviruses were detected in 4 of 7 SOT patients who had no other source of fever identified: 1 had HHV-6B, 1 had CMV, and 2 had both virues (Figure). One of these children was ill and had a high fever and pleuritis with no other pathogens identified. HHV-6A and HHV-7 were not detected in any of the cancer or SOT patients. In our study, frequencies of HHV-6B were similar in transplant recipients and cancer patients (3 of 9 vs. 4 of 30, p = 0.19).

HHV-6B was detected more frequently in children with leukemia and solid-organ transplants than in children with other cancers. This finding may be the result of similarities in the degree and type of immune suppression. Children with leukemia and SOT patients generally receive intense initial immune suppression followed by a constant lower level of suppression. In contrast, solid-tumor patients generally receive monthly immune-suppressive treatments and seldom have prolonged lymphopenia. HHV-6B DNAemia was more frequent in children sampled during the first 6 months of immune suppression when immune suppression is most intense, which is consistent with findings for pediatric bone marrow and solid-organ transplant recipients (*34*). Detection of HHV-6B early after initiation of therapy in patients with cancer (Figure) suggests that the presence of virus DNA may be related to intense immune suppression and not to random occurrence, which would not have clustered early in therapy.

Our observations of possible clinical associations with β-herpesvirus infection concur with those of Michalek et al. (*23*,*25*) of a cohort dating from 1993 to 1999. Our population included 1 child with suspected HHV-6 disease and hepatitis, which has been seen in association with HHV-6 activity after liver (*35*), renal (*36*), and stem cell transplantation (*37*).

In contrast to laboratory detection for CMV, testing for HHV-6 and HHV-7 DNA is often conducted by using in-house assays, which makes comparison across studies difficult. We used an assay for detecting HHV-6 DNA that is commercially available in Europe. This test was concordant with our in-house assay in detecting previously characterized HHV-6 isolates (D. Kohn et al., unpub. data). In addition to standardization of molecular testing, various methods for detecting HHV-6 during febrile episodes should be evaluated because some investigators have suggested that viral isolation by culture may be more useful in correlating active infection with clinical disease (*38*). Furthermore, reverse transcription–PCR can distinguish active replication from latency (*39*,*40*).

Several limitations are inherent to our cross-sectional study design. First, capturing data at single time points limits the ability to assess the temporality of the virologic burden in relation to disease. Second, although we found HHV-6B positivity during febrile events in 18% of our population, we do not have data during periods without fever and therefore cannot be certain that viremia is specifically associated with disease. The studies by Michalek et al. also included treatment-phase blood specimens that were obtained only during febrile illness or other clinical events suggestive of viral infection (*23*,*25*). Thus, activity of HHV-6 and other lymphotropic viruses during nonfebrile periods in this patient population remains unknown. We studied a heterogeneous patient population that included several types of cancer; the level of immune suppression varied widely. Finally, identification of possible causes of febrile episodes was limited by the assays we performed and the tests ordered by the primary medical team. Thus, we cannot exclude other viral pathogens such as human metapneumovirus or Epstein-Barr virus for which no testing was conducted.

Evidence for our suspected associations between HHV-6B and the febrile events includes 1) our use of

relatively small volumes of blood in the PCR analyses, which reduces the chance of detecting sporadic latently infected lymphocytes; 2) the temporal association of detection of HHV-6B with initiation of chemotherapy; and 3) a lack of other identified sources for the fever. With the exception of the transplant recipient with possible HHV-6B germline integration, HHV-6B or CMV are plausible causes of the febrile episode in every instance in which they were detected.

Our study differs from prior studies in that we assayed for all 4 β-herpesviruses and considered the timing of viral activity relative to the onset of immune suppression. We included solid-organ transplant patients as a reference for cancer patients, and excluded stem cell transplant patients, a population in whom reactivation of CMV and HHV-6 has been studied. By enrolling children with a variety of malignancies, we surveyed a diverse cancer population.

Although the number of patients we studied was small, CMV and HHV-6B are clearly active in pediatric cancer patients. The lack of other identified agents coincident with fever suggests that these viruses may cause febrile illness in this population. Our observations, combined with those of Michalek et al. (*23*,*25*), suggest that HHV-6B and CMV should be included in the differential diagnoses of febrile disease in children with cancer. Further investigation in this patient population is needed to clarify the role of β-herpesviruses in these febrile illnesses.

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Seroprevalence and Risk Factors for Human Herpesvirus 8 Infection, Rural Egypt[1]

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To determine whether human herpesvirus 8 (HHV-8) is associated with schistosomal and hepatitis C virus infections in Egypt, we surveyed 965 rural household participants who had been tested for HHV-8 and schistosomal infection (seroprevalence 14.2% and 68.6%, respectively, among those <15 years of age, and 24.2% and 72.8%, respectively, among those ≥15 years of age). Among adults, HHV-8 seropositivity was associated with higher age, lower education, dental treatment, tattoos, >10 lifetime injections, and hepatitis C virus seropositivity. In adjusted analyses, HHV-8 seropositivity was associated with dental treatment among men (odds ratio [OR] 2.4, 95% confidence interval [CI] 1.1–5.2) and hepatitis C virus seropositivity among women (OR 3.3, 95% CI 1.4–7.9). HHV-8 association with antischistosomal antibodies was not significant for men (OR 2.1, 95% CI 0.3–16.4), but marginal for women (OR 1.5, 95% CI 1.0–2.5). Our findings suggest salivary and possible nosocomial HHV-8 transmission in rural Egypt.

Human herpesvirus 8 (HHV-8, also called Kaposi sarcoma [KS]–associated herpesvirus) is the infectious cause of KS (*1*) and is prevalent in Africa (*2*). HHV-8 seroepidemiology parallels imperfectly KS epidemiology (*3*). Adult HHV-8 seropositivity is very high in eastern and central Africa (70%–90%), where KS is endemic, and lower in southern and northern Africa (10%–40%), including Egypt, where KS is more rare (*4*). This variation may be due, in part, to socioeconomic or environmental factors (*5*) influencing HHV-8 transmission or pathogenesis. HHV-8 is transmitted through contact with saliva (*6*–*8*), but sexual (*9*) and blood-borne (*10*) transmission also occur.

HHV-8 seroepidemiology in Egypt is incompletely described (*3*,*11*,*12*). Egypt offers the opportunity to investigate how HHV-8 correlates in the general population with well-characterized hepatitis C virus (HCV) (*13*) and schistosomal infections (*14*). Hundreds of thousands of Egyptians were exposed to multiple intravenous injections during treatment campaigns to control schistosomiasis from the 1950s until 1982, which resulted in an epidemic of HCV (*15*). Schistosomal infection has been reported to suppress the immune response to HCV, which could lead to more persistent infections in those who are co-infected (*16*–*20*). Whether a similar relationship exists with HHV-8 is not known. The rarity of KS in Egypt, despite its occurrence in organ transplant recipients (*21*), suggests that schistosomal-induced immunosuppression may not increase KS risk substantially. We sought to test the hypothesis that schistosomal seropositivity is associated with HHV-8 seropositivity, which would support the concept that schistosomal-induced immunosuppression modulates susceptibility to HHV-8 infection.

Methods

Patient Selection and Serologic Testing for HHV-8 and Schistosomal Infections

We randomly selected residual frozen serum samples from 965 of ≈6,000 persons who had participated in the HCV and schistosomiasis epidemiologic, population–based, household survey in Assiut Governorate, in rural southern Egypt in 1992 (*13*). Adults and parents of children

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[1]Results were presented, in part, at the 9th International Workshop on Kaposi's Sarcoma–associated Herpesvirus (KSHV) and Related Agents, Cape Cod, Massachusetts, USA July 12–15, 2006.

≤15 years of age who had participated in the original survey gave informed consent and answered interviewer-administered questions about demographics, socioeconomic status, medical treatment, and parenteral exposures, including injections, transfusions, operations, dental treatment, and tattoos. Each participant gave a venous blood sample for serologic testing (*13*). The proportion of children included in our study was slightly lower than in the original survey because serum samples from some children had been exhausted by tests for HCV and other hepatitis viruses. However, the included children were otherwise representative of the original survey population.

Anti–HHV-8 antibodies were measured by using an enzyme immunoassay to K8.1 glycoprotein (a lytic-phase antigen) as previously reported (*22*,*23*). Antischistosomal antibodies were measured by using enzyme-linked immunoelectrotransfer blots (EITB) to detect species-specific antibodies against microsomal glycoprotein antigens from *Schistosoma hematobium* and *S. mansoni* (98% sensitivity and 99% specificity) (*24*). *S. hematobium* is the predominant species causing infection in Assiut Governorate; concurrent or single infection with *S. mansoni* among local inhabitants is rare (*14*).

Statistical Methods

Because no standard for testing for HHV-8 infection exists, we previously based cutoff values for defining seropositivity on visual inspection of the distribution of the K8.1 assay optical density (OD) values (*10*), assuming that there are seronegative and seropositive subpopulations. However, this approach is subjective. To more objectively define seropositivity, we applied mixture models to the OD data (*25*). Briefly, the mixture model was based on the assumption that the OD value for each participant arises from either a seronegative or a seropositive subpopulation. The formulas and details on parameters for the statistical model are described elsewhere ([*25*]; online Appendix, available from www.cdc.gov/EID/content/14/4/586-app.htm). We assumed that when the calculated posterior probability of seropositivity was ≥0.5, then the person was seropositive. In sensitivity analyses (online Appendix), we used alternative parameters of the model and also excluded persons with an intermediate posterior probability (range: 0.4–0.6).

After defining seropositivity, we used logistic regression models (PROC GENMOD, SAS 8.0; SAS, Cary, NC, USA) to calculate odds ratios (ORs) for HHV-8 associations with demographic, behavioral, and clinical risk factors. We used generalized estimation equations that accounted for correlations between persons living in the same household to calculate 95% confidence intervals (CIs) (*26*). Because HHV-8 seropositivity is age dependent and modes of transmission may differ between children and adults, we performed univariate and multivariable analyses separately for children (<15 years of age) and adults (≥15 years of age). Because we postulated a priori that antischistosomal antibodies were associated with HHV-8, we included schistosomal status in all multivariable models. To adjust for potential confounding, we included in our multivariable models those variables that were associated with HHV-8 seropositivity at $p \leq 0.1$ in univariate analyses. Age was fitted with a trend whenever this resulted in a statistically significantly improved model fit; otherwise, it was fitted as a categoric variable with dummy values. Two-tailed p values ($p<0.05$) were considered statistically significant, while p values between 0.1 and 0.05 were suggestive of a trend.

Results

None of the original household survey participants had a history of KS (*13*). HHV-8 seroprevalence was lower among children compared with adults (14.3% vs. 24.2%, $p<0.001$). Among children, in unadjusted analyses, HHV-8 seroprevalence was higher in girls than boys (20% vs. 9%; OR 2.4, 95% CI 1.1–5.3). HHV-8 seroprevalence was not significantly elevated in children with a history of ≥10 lifetime injections compared with those with <10 lifetime injections (18% vs. 11%, OR 1.8, 95% CI 0.8–3.8) nor among those with schistosomal antibodies (16% vs. 10%; OR 1.7, 95% CI 0.7–4.3). Age, education, dental treatment, tattoos, and HCV antibodies were unrelated to HHV-8 seropositivity among children.

Among adult men and women combined, unadjusted analyses showed that HHV-8 seropositivity was higher among older participants (≥45 years of age) compared with younger participants (15–24 years of age; OR 4.1, 95% CI 2.6–6.6); among those currently married (OR 1.9, 95% CI 1.2–3.0) or divorced, separated, or widowed (OR 3.3, 95% CI 1.7–6.4) versus never married; and among those with a history of dental treatment (OR 2.1, 95% CI 1.5–2.9), ≥10 lifetime injections (OR 1.5, 95% CI 1.0–2.3), tattoos (OR 1.7, 95% CI 1.1–2.7), or HCV seropositivity (OR 1.8, 95% CI 1.0–3.3) compared with participants without these characteristics. Conversely, HHV-8 seropositivity was lower among adults who reported primary (OR 0.6, 95% CI 0.4–1.0) or higher level of formal education (OR 0.3, 95% CI 0.2–0.6) compared with participants without formal education.

Antischistosomal antibodies, which indicate past as well current infection, were detected among 72.8% of participants. Current infection, as indicated by ova in stool or urine samples, was noted for 4% of participants, all of whom had *S. hematobium*. *S. mansoni* was only recently introduced in Assiut Governorate and remains rare and focal in distribution (*14*). Almost all participants who had *S. mansoni* antibodies were also positive for *S. hematobium*. Patterns of schistosomal seropositivity differed between women and men. Among women, antischistosomal antibody prevalence

was inversely related with age (68.8% in those 15–24 years of age vs. 34.1% in those ≥45 years of age; p<0.001); was lower in those who were widowed, divorced, or separated compared with those who were married or who had never married (38.8% vs. 63.4%; p = 0.003); but was unrelated to education (p = 0.36) or HCV seropositivity (p = 0 .12). Among men, antischistosomal antibody prevalence was unrelated to age (p = 0.61), marital status (p = 0.73), education (p = 0.64), or HCV seropositivity (p = 0.12).

In unadjusted sex-specific analyses (Tables 1 and 2), HHV-8 seropositivity was not associated with antischistosomal antibodies in women (OR 1.0, 95% CI 0.6–1.5); it was not significantly associated in men (OR 2.3, 95% CI 0.3–19.0), because only 9 men had no antischistosomal antibodies. The HHV-8 associations with age, formal education, marital status, and history of dental treatment among men and women combined were largely recapitulated in the sex-specific analyses, except for associations with having tattoos, ≥10 lifetime injections, and HCV seropositivity, which were evident in women but not men (Tables 1 and 2). HHV-8 was significantly associated with cigarette smoking; cigarette smoking was only recorded for men.

Table 1. Prevalence and crude OR of association of HHV-8 seropositivity with demographic, behavioral, and clinical characteristics among male patients, Egypt*

Characteristic	n/N	%	OR	95% CI	p value†
Total	52/235	22.1	–	–	–
Age group, y					<0.001
15–24	8/96	8.3	Ref	–	–
25–34	16/54	29.6	4.6	2.0–11.0	–
35–44	10/30	33.3	5.5	1.9–15.7	–
≥45	18/55	32.7	5.3	2.2–13.2	–
Education					0.001
None	19/50	38.0	Ref	–	–
Primary	22/100	22.0	0.5	0.2–1.0	–
Secondary/postsecondary	11/85	12.9	0.2	0.1–0.6	–
Job					0.001
Student	2/38	5.3	Ref	–	–
Not working	6/31	19.3	4.3	0.8–23.3	–
Farmer	25/87	28.7	7.3	1.6–32.4	–
Trade, service, production	13/63	20.6	4.7	1.1–20.0	–
Technician/secretary	6/16	37.5	10.8	1.9–61.3	–
Marital status					0.004
Not married	11/91	12.1	Ref	–	–
Married	39/136	28.7	3.1	1.4–6.5	–
Separated/divorced/widowed	2/8	25.0	7.6	1.0–60.8	–
Dental treatments					<0.001
No	16/128	12.5	Ref	–	–
Yes	36/107	33.6	3.5	1.8–7.1	–
Tattoos					0.53
No	50/220	22.7	Ref	–	–
Present	2/15	13.3	0.5	0.1–2.5	–
HCV serostatus					0.72
Negative	48/214	22.4	Ref	–	–
Positive	4/21	19.0	0.8	0.3–2.5	–
Injections (lifetime)					0.74
<10	20/72	20.8	Ref	–	–
≥10	37/163	22.7	1.1	0.6–2.1	–
Smoking					0.02
Never	23/138	16.7	Ref	–	–
Ever	29/97	29.9	2.1	1.1–4.0	–
Goza‡					0.77
Never	42/193	21.8	Ref	–	–
Ever	10/42	23.8	1.1	0.5–2.4	–
Schistosomiasis					0.43
Negative	1/9	11.1	Ref	–	–
Positive	51/226	31.0	2.3	0.3–19.0	–

*OR, odds ratio; HHV-8, human herpesvirus 8; CI, confidence interval; Ref, referrent; HCV, hepatitis C virus.
†p value for age group and education is test for trend; otherwise, p value is test for heterogeneity
‡Goza is a method of tobacco smoking in which tobacco smoke passes through a water pipe.

Table 2. Prevalence and crude OR of association of HHV-8 seropositivity with demographic, behavioral, and clinical characteristics among female patients, Egypt*

Characteristic	n/N	%	OR	95% CI	p value†
Total	125/495	25.3	–	–	–
Age group, y					<0.001
15–24	23/148	15.5	Ref	–	–
25–34	21/106	19.8	1.4	0.7–2.7	–
35–44	31/115	27.0	2.0	1.1–3.6	–
≥45	50/126	39.7	3.6	2.1–6.3	–
Education					<0.001
None	88/307	28.7	Ref	–	–
Primary	34/154	32.7	0.7	0.4–1.1	–
Secondary/postsecondary	4/38	22.5	0.3	0.1–0.8	–
Job					0.11
Other‡	2/21	9.5	Ref	–	–
Housewife	121/470	25.7	3.3	0.8–14.3	–
Dental treatments					0.02
No	49/239	20.5	Ref	–	–
Yes	77/260	29.6	1.6	1.1–2.5	–
Tattoos					0.006
No	93/410	22.7	Ref	–	–
Present	33/89	37.1	2.0	1.2–3.3	–
HCV serostatus					0.006
Negative	112/470	23.8	Ref	–	–
Positive	14/29	48.3	3.0	1.4–6.5	–
Injections (lifetime)					0.03
<10	23/128	18.0	Ref	–	–
≥10	103/371	27.8	1.7	1.1–2.9	–
Schistosomiasis					0.94
Negative	48/189	25.4	Ref	–	–
Positive	76/303	25.1	1.0	0.6–1.5	–

*OR, odds ratio; HHV-8, human herpesvirus 8; CI, confidence interval; Ref, referrent; HCV, hepatitis C virus.
†p value for age group and education is test for trend; otherwise, p value is test for heterogeneity.
‡Job category for women: "Other" includes women who are students, are not working, or have technical or scientific jobs.

In a multivariable analysis, HHV-8 seropositivity was higher among girls than boys (OR 2.6, 95% CI 1.2–5.8) and marginally associated with antischistosomal antibodies (OR 2.2, 95% CI 0.8–5.6). Among adult men, HHV-8 seropositivity was independently associated with older age and history of dental treatment but not with schistosomal antibodies (OR 2.3, 95% CI 0.3–16.1; Table 3). Among women, HHV-8 seropositivity was associated with older age, HCV seropositivity, and marginally with antischistosomal antibodies (OR 1.5, 95% CI 1.0–2.5; p = 0.07).

Discussion

We report HHV-8 seroepidemiology in a rural population in Egypt in which correlates of schistosomal and HCV were previously characterized (*13*,*14*). As in other populations, HHV-8 seropositivity in Egypt rose with increasing age (*3*,*5*). In subgroups, we found associations of HHV-8 seropositivity with history of dental therapy, lifetime injections, tattoos, and HCV seropositivity.

Previous studies of HHV-8 in Egypt (*11*,*12*) reported a seroprevalence of ≈40%, which is ≈2× the prevalence we observed. Those studies were hospital based, were conducted in urban areas, and detected anti-HHV-8 antibodies with lytic immunofluorescence assays; all of these factors may have contributed to higher prevalence estimates. Despite these differences, the patterns in our associations support their validity. First, we detected HHV-8 antibodies in children, consistent with earlier reports and nonsexual HHV-8 spread in Egypt (*11*). Second, HHV-8 seroprevalence increased with age, in accord with the general pattern of HHV-8 observed in other populations (*5*). Among men, HHV-8 seropositivity was significantly associated with dental treatment, which may be a marker for transmission through saliva- or blood-contaminated dental instruments. Our HHV-8 associations with a history of ≥10 lifetime injections, tattoos, and HCV seropositivity also point to possible blood-borne transmission and agree with other studies (*10*,*27*,*28*). Sexual transmission might be suggested by our HHV-8 association with marital status, but this association did not persist after adjustment for age. Rather than ongoing transmission among adults, higher HHV-8 seropositivity in older persons may be due to a birth-cohort effect, i.e., reflecting periods of elevated HHV-8 transmission risk in the past. In this regard, the widespread use of intravenous injections for schistosomiasis treatment and control programs from 1950 to 1982 would be consistent with a

Table 3. Adjusted OR of association of HHV-8 seropositivity with demographic and clinical variables among adults, Egypt*

Characteristic	Men			Women		
	OR	95% CI	p value	OR	95% CI	p value
Age group, y†			0.002			
15–24	Ref	–	–	Ref.	–	–
25–34	1.6	1.2–2.2	–	0.8	0.4–1.6	0.53
35–44	2.6	1.4–4.9	–	1.5	0.8–2.9	0.15
≥45	4.3	1.7–11.0	–	3.1	1.5–6.4	<0.001
Dental treatments‡			0.04			
No	Ref	–	–	–	–	–
Yes	2.3	1.1–4.9	–	–	–	–
HCV serostatus‡			–			0.007
Negative	–	–	–	Ref	–	–
Positive	–	–	–	3.3	1.4–7.9	–
Schistosomiasis§			0.47			0.07
Negative	Ref	–	–	Ref	–	–
Positive	2.3	0.3–16.1	–	1.5	1.0–2.5	–

*OR, odds ratio; HHV-8, human herpesvirus 8; CI, confidence interval; Ref, referrent; HCV, hepatitis C virus.
†p value is for age group fitted with trend among men; p values for heterogeneity for categories given for women (see Statistical Methods).
‡Missing values in sex-specific analyses mean the variable was not significant and was excluded from final multivariable model.
§Schistosomiasis seropositivity was included in models even when not significant because we hypothesized a priori that it was associated with HHV-8 seropositivity (see online Appendix, available from www.cdc.gov/EID/content/14/4/586-app.htm).

birth-cohort effect for high HHV-8 seroprevalence among our older participants. However, most populations, including those with no similar historical programs, have higher HHV-8 seroprevalence among older persons, which suggests that ongoing HHV-8 transmission among adults is a more likely explanation.

We found a 2-fold higher HHV-8 seroprevalence in persons who also had schistosomal antibodies. In multivariable analyses, the CI for this association was 1.0–2.5 among women, but it was wide among children (0.8–5.6) and especially among men (0.3–16.1). Chance association and serologic cross-reactivity between HHV-8 and schistosomal antibodies are possible explanations, but schistosomal antibodies may be a valid marker for exposure to injections in the historical anti-schistosomal program; these findings are consistent with parenteral transmission of HHV-8 as discussed above. Another possibility is that anti-schistosomal antibodies may be a marker for contact with surface water sources or walking barefoot, which are also risk factors for HHV-8 seropositivity and KS (*5*,*7*,*29*). No biologic explanation has been advanced for these environmental correlations with HHV-8 and KS. Our study suggests that perhaps contact with surface water or walking barefoot is a marker of exposure to and potential infection with *Schistosoma* or other water-related parasites. Infection with such parasites could influence the natural history of HHV-8 by shifting the immune response from a T helper 1 (Th1)–type response, which is central to controlling viral infections, to a Th2-dominant response (*30*), which is less effective against viral infections. If this model is correct, schistosomal infection could increase susceptibility to HHV-8 infection at relatively low exposure to the virus. In parallel, Th2-dominant hosts may fail to effectively control HHV-8 infection and thus shed infectious virions in saliva more frequently and at higher levels, resulting in higher HHV-8 transmission. If our findings are confirmed, they could drive investigations of environmental characteristics, including exposures to volcanic soil or plants (*31*), to explain variation in HHV-8 infection and possibly KS.

Our study has several limitations. First, current HHV-8 serologic assays have imperfect specificity and sensitivity (*32*), which could have contributed to the lower HHV-8 seroprevalence observed. Except for the possible cross-reactivity mentioned above, serologic misclassification is likely to be random, which would attenuate associations toward the null. Second, our HCV and schistosomal antibody assays cannot distinguish current from resolved infections, diminishing the strength of observed associations as well. Third, with our cross-sectional design, we cannot determine the temporality of associations. This limitation may be particularly relevant to our findings of HHV-8 with antischistosomal antibodies. The antischistosomal programs surely reduced the prevalence and load of schistosoma eggs, but they may also have contributed to HHV-8 transmission through injections. Finally, we studied only ≈15% of the participants in the original survey, which limited our statistical power to estimate some associations.

The strengths of our study include our state-of-the-art serologic methods, our model-based approach to estimating infection risk, and our well-characterized general population with detailed socioeconomic and clinical data. HHV-8 seropositivity was associated with older age, dental therapy, lifetime injections, and HCV and schistosomiasis seropositivity. These findings suggest salivary and possible nosocomial HHV-8 transmission in rural Egypt and a potential biologic explanation for geographic variation of HHV-8 seropositivity and KS.

Dr Mbulaiteye is a tenure-track investigator in the Infections and Immunoepidemiology Branch (formerly Viral Epidemiology Branch), Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland, USA. He is interested in characterizing the relationship between immunity, human herpesvirus infection, and cancer, particularly in Africa.

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Retrospective Analysis of Monkeypox Infection

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Serologic cross-reactivity between orthopoxviruses is a substantial barrier to laboratory diagnosis of specific orthopoxvirus infections and epidemiologic characterization of disease outbreaks. Historically, time-consuming and labor-intensive strategies such as cross-adsorbed neutralization assays, immunofluorescence assays, and hemagglutination-inhibition assays have been used to identify orthopoxvirus infections. We used cross-adsorption to develop a simple and quantitative postadsorption ELISA for distinguishing between monkeypox and vaccinia infections. Despite the difficulty of diagnosing clinically inapparent monkeypox in previously vaccinated persons, this technique exhibited 100% sensitivity and 100% specificity for identifying clinically overt monkeypox infection irrespective of vaccination history. We also describe a Western blot technique in which up to 3 diagnostic bands may be used to distinguish between vaccinia and monkeypox infection. The techniques described provide independent diagnostic tests suitable for retrospective analysis of monkeypox outbreaks.

Human monkeypox is a zoonotic disease found in remote areas of western and central sub-Saharan Africa and is an important public health issue in these areas (*1*,*2*). Clinical symptoms of monkeypox can be similar to those of chickenpox (caused by varicella-zoster virus), and these symptoms can cause difficulties in diagnosing cases on the basis of clinical symptoms alone (*2*,*3*). These symptoms can also vary among persons; most American patients in the 2003 monkeypox outbreak had a rash with 1 to >100 skin lesions (*4*–*7*), whereas others may have monkeypox infection without exanthem (*8*,*9*). Moreover, adults with preexisting immunity from childhood smallpox vaccinations may experience milder symptoms (*5*,*6*,*10*) or no symptoms (*6*). Standard clinical algorithms (*11*,*12*) may fail to identify these mild or asymptomatic cases; likewise, they are difficult to confirm by virologic methods because of the absence of skin lesions. Because of the extensive variability in clinical symptoms and common misdiagnosis as chickenpox, developing multiple diagnostic techniques that can be used to identify and confirm monkeypox is crucial.

We describe 2 serologic techniques for diagnosing monkeypox infection. These techniques were based on diagnostic approaches such as radioimmunoassays and neutralization assays that used a preadsorption step to remove or reduce cross-reactive orthopoxvirus antibodies before detection of species-specific antiviral antibodies (*13*–*15*). These basic ELISA and Western blot methods will be useful for orthopoxvirus-specific serosurveys and retrospective analysis of monkeypox outbreaks.

Materials and Methods

Participants

Adults previously characterized as having suspected, probable, or confirmed cases of monkeypox during the 2003 Wisconsin monkeypox outbreak provided informed written consent and completed a medical history questionnaire before participation in our previous study (*6*). Monkeypox confirmation was based on the standard case definition and confirmation by the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA, during the outbreak (*4*) or by subsequent immunologic tests (*6*). Participants were grouped according to vaccination history (Table). Samples from 13 unvaccinated monkeypox-immune persons and 8 vaccinated monkeypox-immune persons were assessed. Of the 8 vaccinated persons, 3 had clinically inapparent cases and were not aware of having been infected with monkeypox because of no noticeable disease symptoms (*6*). However, these are described as laboratory-confirmed cases of monkeypox on the basis

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Table. Comparison of monkeypox-specific diagnostic tests*

Patient no.	Serologic techniques		Cellular techniques	Direct viral detection	Postadsorption ELISA	Postadsorption Western blot†		
	Paired IgG	Peptide ELISA				39 kDa	124 kDa	148 kDa
Monkeypox								
447	C	C	C	C	C	+	+	+
452	C	C	C	C	C	+	+	+
453	C	C	C	P/S	C	+	+	+
461	C	C	C	NA	C	+		+
462	C	C	C	C	C	+		
481	C	C	C	NA	C	+	+	
482	C	C	C	NA	C	+	+	
484	C	C	C	NA	C	+		
489	C	C	C	NA	C	+	+	
473	C	C	C	NA	C	+	+	
519	C	C	C	C	C	+	+	
520	C	C	C	C	C	+	+	
557	ND	ND	ND	NA	C	+	+	+
Vaccinia–monkeypox								
446‡	C	C	C	ND	U			
449‡	C	C	U	ND	U	+		
450	C	C	C	P/S	C	+		
451	C	C	C	C	C	+		
454	C	C	C	P/S	C	+	+	+
455‡	C	C	C	ND	U	+		
463	C	C	C	C	C	+		
500	C	C	C	ND	C			+

*These studies are based on persons who were infected with monkeypox during the 2003 outbreak in Wisconsin and who were tested by independent laboratories by using serologic (*6*), cellular (*6*), and virologic (*4*) techniques. Serologic techniques included identification of monkeypox peptide-specific immunoglobulin (Ig) G by peptide ELISA and kinetic analysis of IgG to orthopoxvirus in paired plasma samples (2–4 mo and 1 y postinfection) by endpoint ELISA. Orthopoxvirus-specific CD8+ T cells were measured by using intracellular cytokine staining analysis. Plasma samples were obtained 2–4 mo postinfection, except for patients 473 and 500 (6 mo), 519 and 520 (12 mo), and 557 (30 mo). Virologic confirmation was obtained by viral culture, PCR, electron microscopy, and immunohistochemical analysis of tissue samples (*4*). C, confirmed; P/S, probable/suspected; NA, not available; ND, not done; U, unconfirmed.
†A+ indicates that at least 5/6 analysts scored the band of interest as positive.
‡Clinically inapparent monkeypox infection in previously vaccinated persons as determined by cellular and serologic retrospective diagnostic techniques (*6*).

of multiple immunologic assays (*6*) (Table). Control participants included recent primary smallpox vaccinees (n = 10), revaccinated persons (n = 10) examined 2–4 months postvaccination, long-term immune smallpox vaccinees examined 20–40 years postvaccination (n = 10), and unvaccinated orthopoxvirus-naive persons (n = 12). Heparinized blood was centrifuged over Histopaque-1077 (Sigma, St. Louis, MO, USA), and plasma was collected and stored at –80°C. Studies involving human participants were reviewed and approved by the Institutional Review Board for Oregon Health and Science University.

Viruses and Cells

Monkeypox virus (Zaire strain) and vaccinia virus (Western Reserve strain) were grown in BSC40 cells by using a multiplicity of infection of 0.1 and harvested at 48 h postinfection. Cells were lysed by 3 freeze/thaw cycles in 10 mmol/L Tris, pH 8.0, and used as a virus lysate for preadsorption in ELISA and Western blots. Where indicated, monkeypox and vaccinia viruses were also purified by ultracentrifugation through 36% sucrose at 40,000 × *g* for 80 min, followed by band extraction after 25%–40% sucrose gradient sedimentation (33,000 × *g* for 40 min). Protein concentration was measured by the modified Lowry assay (Pierce, Rockford, IL, USA). Purified virus was inactivated for 2 h at 56°C; viral lysates were inactivated with 3% H_2O_2 at room temperature for 2 h. Uninfected BSC40 cell lysate was treated similarly to the infected cells described above.

Postadsorption ELISA

High protein-binding ELISA plates (Corning-Costar, Corning, NY, USA) were coated with an optimized concentration of H_2O_2-inactived monkeypox-infected BSC40 cell lysate. Plasma samples were preadsorbed with equivalent (6×10^8 PFU/mL) concentrations of H_2O_2-inactived monkeypox or vaccinia whole-cell lysate at a 1:30 dilution (5 μL plasma in 145 μL viral lysate) for 30 min at 37°C. Nonadsorbed samples were similarly treated with ELISA blocking buffer (phosphate-buffered saline containing 5% nonfat dry milk and 0.05% Tween 20). Samples were then added directly to ELISA plates, serially diluted in blocking buffer, and incubated at room temperature for 1 h. As a precaution against human blood-borne pathogens, samples

were then treated with 3% H_2O_2 (final concentration) for an additional 30 min. After washing, horseradish peroxidase–conjugated mouse antihuman immunoglobulin (Ig) G monoclonal antibody (clone G18–145; BD Pharmingen, San Diego, CA, USA) was added to the wells. Plates were washed after 1 h and detection reagents were added. Substrate was prepared (*o*-phenylenediamine; Sigma-Aldrich, St. Louis, MO, USA) and diluted to a concentration of 0.4 mg/mL in 0.05 M citrate, pH 5.0, and H_2O_2 was added (final concentration of 0.01%). Color was developed for at least 20 min before the reaction was stopped by adding 1 M HCl; plates were read at 490 nm.

Antibody titers were determined by log-log transformation of the linear portion of the dilution curve with 0.1 optical density units used as the endpoint, and transformation was performed on final values (*6*). For an Excel (Microsoft, Redmond, WA, USA) file containing a template of these calculations, please contact the corresponding author, or see the example given in the online Technical Appendix (available from www.cdc.gov/EID/content/14/4/592-Techapp.pdf).

Differential Western Blot

Western blot procedures were performed with the following modifications. Two micrograms of gradient-purified monkeypox or vaccinia virus was separated by 4%–20% Tris-glycine gradient sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, CA, USA) under reducing conditions. Equivalent protein loading was confirmed on representative gels by staining with GelCode Blue (Pierce).

Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Pierce), and membrane strips with 3 lanes containing a molecular mass standard (SeeBlue Plus 2; Invitrogen), monkeypox, and vaccinia were blocked with phosphate-buffered saline containing 1% Tween 20 and 5% nonfat dry milk. Plasma was diluted 1:20 in uninfected cell lysate or H_2O_2-inactived vaccinia lysate (adjusted to a concentration of 2.5 mg/mL total protein) for 30 min at 37°C. Adsorbed plasma was adjusted to a 1:10,000 dilution in 10 mL of blocking buffer and incubated with membranes overnight in 50-mL conical tubes at 4°C with rocking. After 3 washes in blocking buffer, reactive bands were identified with horseradish peroxidase–conjugated goat antihuman IgG (γ chain specific; Jackson ImmunoResearch, West Grove, PA, USA) by using chemiluminescent detection (SuperSignal West Dura Substrate; Pierce). Plasma from the same monkeypox patient was used as a positive control in each experiment to identify the position of diagnostic bands. Blots were exposed to x-ray film until diagnostic bands were clearly visible, and other films were then overexposed to ensure that any low-intensity bands were given ample opportunity to appear. Plasma from some orthopoxvirus-naive persons did not react with any monkeypox or vaccinia protein bands. In these instances, films were exposed 10× longer than the last readable positive control exposure before a negative result was recorded. Films were scanned and the positions of diagnostic bands were indicated on the basis of the positive control. Analysts scored the vaccinia-preadsorbed Western blots for the 39-kDa, 124-kDa, and 148-kDa diagnostic bands as present only in the monkeypox lane, absent from the monkeypox lane, present in the monkeypox and vaccinia lanes (i.e., experimental equivocal), or technical equivocal caused by nonspecific background. Immunoreactive bands deemed experimental equivocal were counted against the sensitivity or specificity of the assay. Blots containing technical equivocal data were repeated, and analysts rescored bands that were unreadable in the initial screen before determining final sensitivity and specificity.

Results

Postadsorption ELISA

Orthopoxviruses have highly conserved genomes (*16*), which results in high levels of antibody cross-reactivity. However, viruses such as monkeypox also contain genes that are absent, mutated, or truncated in vaccinia; these gene products can be used to distinguish between monkeypox and vaccinia infections (*6,17–21*). The concentration of viral antigen used in the preadsorption step of the ELISA was determined by using high-titer plasma from 2 recently vaccinated vaccinia-immune persons obtained at the peak (day 21) of the anamnestic response with ELISA titers that were ≈10-fold higher than the highest convalescent-phase samples used in the rest of the experiments. Two of the highest titer samples from monkeypox-immune persons (obtained at 2 months postinfection) were also used in these preliminary studies (online Technical Appendix). These samples provided a rigorous test of homologous and heterologous antigens to deplete antiviral antibodies before performing the ELISA. Titration of virus antigen indicated that lysates normalized to contain 6×10^8 PFU equivalents/mL were best for differentiating between vaccinia and monkeypox infections.

Orthopoxvirus-naive persons or persons infected with monkeypox or vaccinia were then tested to establish the diagnostic validity of this approach by using monkeypox-coated ELISA plates (Figure 1, panel A). If plasma from a representative monkeypox-immune person was preincubated with vaccinia antigen, virus-specific antibody titers decreased (from 39,904 ELISA units [EU] to 13,912 EU). However, if the sample was preincubated with an equivalent amount of monkeypox antigen before performing the ELISA, monkeypox antibody titers were further decreased, to 2,542 EU. For the data of the monkeypox-immune

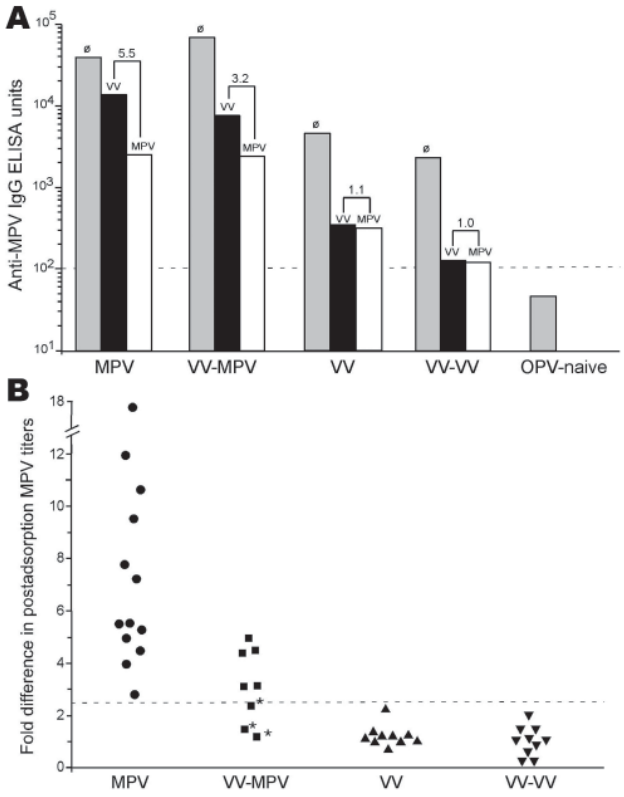

Figure 1. Diagnosis of monkeypox by postadsorption ELISA. Plasma samples were obtained from monkeypox-immune persons (2–30 months postinfection), vaccinia-immune persons (2–4 months postinfection), or uninfected orthopoxvirus-naive persons and tested on ELISA plates coated with inactivated monkeypox antigen. A) A representative monkeypox-specific ELISA with plasma samples from an unvaccinated monkeypox-infected person (MPV), a previously vaccinated (i.e., vaccinia-immune) monkeypox-infected person (VV-MPV), a vaccinia-immune person (VV), a vaccinia-immune person who was revaccinated with vaccinia (VV-VV), and an uninfected orthopoxvirus-naive person (OPV-naive). Plasma was not preadsorbed (∅, gray bars), preadsorbed with inactivated vaccinia antigen (black bars), or preadsorbed with inactivated monkeypox antigen (white bars) before ELISA on monkeypox-coated plates. Numbers above bars refer to differences in postadsorption MPV ELISA titers after adsorption with vaccinia antigen compared with adsorption with monkeypox antigen. Plasma from 1 orthopoxvirus-naive person (representative of n = 12) was not preadsorbed with viral antigen because it was seronegative (<100 ELISA units) and below our detection limit (dashed horizontal line). B) Plasma samples from monkeypox-infected persons (•, n = 13), vaccinia-immune monkeypox-infected persons (■, n = 8), vaccinia-immune persons (▲, n = 10), and revaccinated vaccinia-immune persons (▼, n = 10) were tested by postadsorption ELISA. Data show fold-differences of monkeypox antibody titers after adsorption with vaccinia antigen compared with adsorption with monkeypox antigen. Dashed horizontal line indicates a diagnostic cutoff indicative of a positive result, which was determined as a postadsorption difference score of >2.5. *Denotes results of plasma samples obtained from persons with clinically inapparent monkeypox infection.

person shown in Figure 1, panel A, there was a 5.5-fold difference (13,912 EU divided by 2,542 EU) in the ability of monkeypox antigen to reduce monkeypox-specific antibody levels than vaccinia antigen. For persons who were vaccinia immune but still had contracted clinically apparent monkeypox disease, the difference in postadsorption ELISA titers was typically smaller (3.2-fold difference in the example shown in Figure 1, panel A), but the difference still demonstrated a clear distinction between preadsorption with monkeypox or vaccinia antigens. If a person had been infected with only vaccinia or revaccinated with vaccinia, there was essentially no difference in the ability of monkeypox antigen to deplete antiviral antibodies than an equivalent amount of vaccinia antigen on a monkeypox-coated ELISA plate. In contrast, orthopoxvirus-naive persons were seronegative by ELISA and can be easily distinguished from monkeypox-immune or vaccinia-immune persons because they score below the limits of detection by ELISA (<100 EU) without any preadsorption steps required.

To determine whether the difference in postadsorption ELISA results could be used effectively to distinguish between vaccinia and monkeypox infections, we plotted the results from primary monkeypox-immune persons, previously vaccinated monkeypox immune persons, primary vaccinia-immune persons, and revaccinated vaccinia-immune persons (Figure 1, panel B). We established a diagnostic cutoff of 2.5 by plotting the difference in postadsorption ELISA titers for each group. To establish the diagnostic cutoff, we used persons with laboratory-confirmed cases of primary monkeypox (*6*) as positive controls and vaccinia-immune persons with no evidence of monkeypox infection as negative controls (Figure 1, panel B). The 2.5-fold difference in postadsorption ELISA titers provided the best balance of high sensitivity (100% positive for primary monkeypox) and high specificity (0% positive for vaccinia immune). Other diagnostic cutoff values resulted in decreased specificity or sensitivity when these 2 divergent groups of positive and negative controls were compared. For example, a diagnostic cutoff of 3.0 reduced the sensitivity of the assay to 93% (12/13) for primary monkeypox but did not affect the specificity (100%). Conversely, a diagnostic cutoff of 2.0 did not affect sensitivity (100%) but decreased specificity from 100% to 90% (i.e., 18/20 vaccinia-immune persons were positive). Vaccinia-immune monkeypox patients typically had lower differences in postadsorption ELISA titers. The lower difference suggests that preexisting immunity to vaccinia may have resulted in a lower induction of monkeypox species-specific antibody responses and is consistent with the results of our previous study (*6*).

Using the established 2.5-fold diagnostic cutoff, we achieved 100% (13/13) sensitivity for detecting primary monkeypox infection and 63% (5/8) sensitivity for detecting

monkeypox infection in vaccinia-immune persons in which monkeypox was a heterologous orthopoxvirus infection. Of the 8 vaccinia-immune persons with monkeypox, 3 were clinically asymptomatic but were previously identified by serologic and cellular techniques (*6*). However, these cases are more difficult to diagnose on the basis of serologic analysis only (*6*) and could not be identified by the postadsorption ELISA (Figure 1, panel B; Table). However, if only persons with clinically overt monkeypox infection were included in the analysis, 100% (5/5) of secondary monkeypox infections were identified by this assay (Figure 1, panel B; Table). We also observed 100% specificity; 20/20 vaccinia-immune controls (10 primary and 10 booster smallpox vaccinations) and 12/12 orthopoxvirus-naive persons were negative by this postadsorption ELISA. We report 86% (18/21) overall sensitivity for detecting monkeypox, 100% (18/18) sensitivity for confirming clinically overt monkeypox, and 100% (32/32) specificity with this approach.

Differential Western Blot

To more easily recognize uniquely reactive monkeypox-specific bands by Western blot and simplify interpretation of this serologic diagnostic technique, we added an adsorption step to reduce cross-reactive antibodies to orthopoxvirus. Separation of 2 μg of purified vaccinia and monkeypox by 4%–20% gradient SDS-PAGE resulted in good separation of protein bands across a broad spectrum of molecular masses (Figure 2, panel A) and effective transfer of separated proteins to the polyvinylidene difluoride membrane. Protein-banding patterns of monkeypox virus and vaccinia virus are similar, but a protein band of ≈39 kDa is visible in the monkeypox lane of the GelCode Blue–stained SDS-PAGE that is missing from the vaccinia lane. Incubation of immune plasma in a 20-fold volume excess of virus antigen (1 μL plasma plus 19 μL vaccinia-infected cell lysate at 6×10^8 PFU equivalents/mL) before performing the Western blot effectively reduced the intensity and detection of cross-reactive bands (Figure 2, panel B).

Diagnostic bands with apparent molecular masses of 148, 124, and 39 kDa were identified when monkeypox-immune plasma was used, but were not observed after Western blot analysis with plasma samples from a representative vaccinia-immune person (Figure 2, panel C) or an orthopoxvirus-naive person (Figure 2 , panel D). Because orthopoxvirus-naive persons were seronegative by ELISA (Figure 1, panel A), the Western blot example shown in Figure 2, panel D, required a 1-hour exposure to identify any faint bands after vaccinia antigen adsorption. In contrast, samples from monkeypox-immune and vaccinia-immune persons were exposed to film for 5 s to 2 min for optimal identification of virus-specific banding patterns. We did not observe a correlation between viral protein levels determined by SDS-PAGE (Figure 2, panel A) and immunodominance by Western blot analysis (Figure 2, panel B).

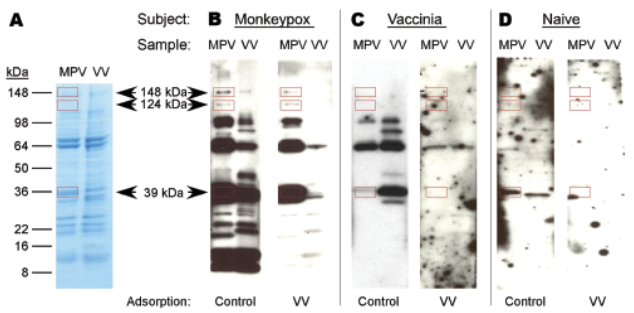

Figure 2. Development of a monkeypox (MPV)–specific diagnostic assay using Western blot analysis. Adsorption of cross-reactive orthopoxvirus antibodies with vaccinia antigen before Western blot analysis provided easier identification of monkeypox-specific bands. A) Two micrograms of sucrose gradient–purified monkeypox virus or vaccinia virus (VV) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4%–20% gels) and stained with GelCode Blue (Pierce, Rockford, IL, USA) to compare banding patterns and confirm equivalent protein loading. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes and probed with plasma from B) a monkeypox-immune person, C) a vaccinia-immune person, or D) an orthopoxvirus-naive person after adsorption of plasma with control antigen (uninfected H_2O_2-treated BSC40 cell lysate) or vaccinia antigen (H_2O_2-inactived vaccinia-infected BSC40 cell lysate). Immunoreactive bands were detected with peroxidase-conjugated antihuman immunoglobulin G plus chemiluminescent substrate and exposed to x-ray film. Arrows indicate location of diagnostic bands with apparent molecular masses of 148, 124, and 39 kDa. Rectangles indicate locations of diagnostic bands.

Unblinded analysis of the Western blots was first performed by 2 independent analysts who had access to patients' medical histories (Figure 3, panel A). Reactivity to the 39-kDa band resulted in 100% sensitivity for identifying primary monkeypox infection and 75% sensitivity for identifying secondary monkeypox infection. However, 20%–30% of the negative control samples (vaccinia primary, vaccinia long-term, and orthopoxvirus naive) also reacted with this band, which resulted in a specificity of only 70%–80% (76% overall specificity). The 124-kDa band showed 96% sensitivity for identifying primary monkeypox infection but only 31% sensitivity for identifying secondary monkeypox infection. However, this protein was more specific than the 39-kDa protein; 0%–10% of negative controls showed reactivity (97% overall specificity). The 148-kDa band showed low diagnostic sensitivity; only 37%–46% of plasma samples from monkeypox-immune persons reacted with it in Western blot analysis. Conversely, the 148-kDa band showed high specificity with 0%–5% of negative control plasma that were positive (i.e., overall specificity >97%). Other bands were also identified as

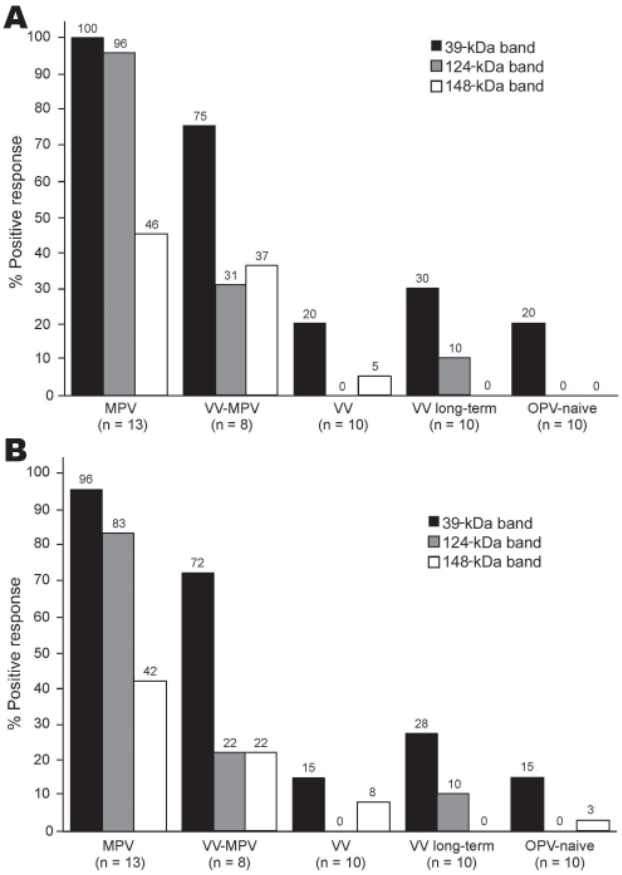

Figure 3. Diagnosis of monkeypox infection by Western blot analysis. Plasma samples from unvaccinated monkeypox-infected (2–30 months postinfection) (MPV), vaccinia-immune monkeypox-infected (2–6 months postinfection) (VV-MPV), primary vaccinia-immune (2–4 months postimmunization) (VV), long-term vaccinia-immune (>20 years postimmunization) (VV long-term), and orthopoxvirus-naive (OPV) persons were analyzed by Western blot after adsorption with vaccinia-infected BSC40 cell lysate to reduce cross-reactive antibodies as described in Figure 2. Immunoreactivity to diagnostic protein bands of ≈39 kDa, 124 kDa, and 148 kDa was assessed by A) unblinded analysts with knowledge of subject medical history (n = 2) and B) blinded analysts who did not have knowledge of subject medical history (n = 4). Findings of each analyst were averaged for each person and percentages shown represent a composite of all data points.

potential diagnostic indicators of monkeypox infection, such as the 98-kDa and 18-kDa bands (Figure 2, panels B and C; online Technical Appendix). However, further analysis showed that the 98-kDa band had low diagnostic potential (48% sensitivity and 72% specificity), as did the 18-kDa band shown in Figure 2, panel B (60% sensitivity and 66% specificity).

Blinded analysts (n = 4) with no knowledge of infection history were asked to score Western blots to determine the feasibility of this approach under conditions in which background clinical information may not be available (Figure 3, panel B). Unblinded analysts reported somewhat higher sensitivity for each of the diagnostic bands and identified the same or slightly lower specificity when interpreting Western blots with vaccinia-immune or orthopoxvirus-naive plasma samples, but the overall results from blinded and unblinded analysis were similar.

Discussion

Historically, several serologic techniques have been used to identify orthopoxvirus infections, including hemagglutination-inhibition (*13*), gel precipitation (*17,22,23*), complement fixation (*22*), cross-neutralization (*14*), immunofluorescence (*24,25*), Western blot (*26*,*27*), radioimmunoassay (*13*,*15*), and ELISA (*28,29*). In previous studies, we used a peptide-based ELISA with peptide sequences from the monkeypox B21R gene to differentially diagnose monkeypox infection (*6*). This approach is highly sensitive and specific for diagnosing monkeypox at 2–4 months postinfection. However, it is yet unknown how quickly peptide-specific antibody responses are mounted or how long they are maintained. Because multiple independent diagnostic techniques should be available for detecting virulent orthopoxvirus infections, we have developed the 2 new assays.

Detection of virus-specific IgM by ELISA is often considered the most useful serologic technique for confirming a recent infection. An IgM capture ELISA for diagnosing monkeypox (*29*) was developed by using vaccinia virus as a surrogate antigen for monkeypox, and the investigators reported 100% specificity and 92% sensitivity. Per CDC monkeypox case definition, these cases had been confirmed by direct viral detection including PCR, electron microscopy, virus isolation, or immunohistochemistry. However, the reported specificity and sensitivity were based on only a subset of samples obtained 5–77 days after onset of rash. Inclusion of samples beyond this range resulted in additional false-negative results and a concomitant decrease in sensitivity. A further limitation of this study is that only virologically confirmed monkeypox cases were included; persons with mild symptoms or vaccine-mediated subclinical infection were not examined.

A more recent study identified 3 unvaccinated contacts who were negative by IgM ELISA but positive by IgG ELISA despite no reported disease symptoms (*10*). If these contacts contracted monkeypox infections, then this would contradict our previous findings in which unvaccinated monkeypox patients showed at least some, if not all, disease symptoms associated with monkeypox (*6,8*). It is difficult to directly compare our results with these other reports (*10,29*) because different subsets of monkeypox patients were examined, different time points were analyzed, and different case definitions were used. For instance, the CDC case definition is based on a combination of clinical,

epidemiologic, and laboratory criteria (www.cdc.gov/ncidod/monkeypox/casedefinition.htm); on the basis of these criteria, monkeypox infection can only be confirmed if virus is detected.

We used epidemiologic criteria (i.e., direct or indirect exposure to monkeypox-infected animals or humans) in addition to laboratory criteria with high sensitivity and specificity (*6*). This approach led to identification of persons in whom monkeypox exposure and disease symptoms occurred, but only immunologic and not virologic laboratory analysis could be performed. Moreover, we identified 3 previously vaccinated persons who were exposed to monkeypox and showed immunologic evidence of infection (*6*). However, because they did not show clinical disease symptoms, they would not meet CDC case definition for monkeypox.

Depletion of cross-reactive antibodies by preadsorption before ELISA enabled the identification of clinically apparent monkeypox infection with 100% sensitivity and 100% specificity. However, 1 limitation was that we were unable to identify clinically inapparent monkeypox infection in previously vaccinated persons (Figure 1, Table). These persons were not tested by virologic methods but were confirmed by immunologic techniques such as early versus late analysis for antibodies to orthopoxvirus, monkeypox B21R peptide ELISA, or orthopoxvirus-specific T-cell analysis (*6*). In our previous study (*6*) and this study, persons with high antiviral immunity from smallpox vaccinations were more likely to mount anamnestic responses to cross-reactive vaccinia epitopes rather than to monkeypox. This may be caused by more limited monkeypox replication, which resulted in fewer disease symptoms (*6*) and less antigenic stimulation of antibody responses to novel virus epitopes. Despite the difficulties involved with serologic diagnosis of clinically asymptomatic cases of monkeypox, the postadsorption ELISA described here provides a robust method for distinguishing between vaccinia and clinically overt monkeypox infections.

Our modified Western blot technique provides an additional independent test for laboratory confirmation of orthopoxvirus infection. It has only modest value as a stand-alone diagnostic test because of <90% sensitivity and specificity overall, but may be useful for confirming cases of monkeypox identified by other virologic or serologic approaches. Relative to standard Western blot approaches (*26*,*27*), preadsorption of plasma with vaccinia antigens decreased the intensity and detection of cross-reactive proteins and enabled easier identification of 3 diagnostic protein bands (39, 124, and 148 kDa). In this assay, 95% (20/21) of monkeypox-immune participants (including 2/3 who had clinically inapparent cases) reacted with at least 1 diagnostic protein band. A cluster of 33 kDa- to 42 kDa-vaccinia proteins was previously described as immunoreactive with pooled human vaccinia immune globulin (*27*). This cluster was also evident in the current study (Figure 2, panels B and C), although detection and intensity of bands within this cluster varied among persons. The 39-kDa monkeypox diagnostic band was readily distinguishable from vaccinia-specific proteins. This band may represent a gene product that is unique to monkeypox, or alternatively it may be common to both viruses but modified in a manner that results in distinct migration characteristics. The immunoreactive protein bands identified were effective in differentiating between monkeypox and vaccinia infection and may prove useful for identifying other virulent orthopoxviruses. Preliminary studies indicate that 5 (83%) of 6 smallpox survivors can be retrospectively diagnosed by using this technique.

Serologic diagnostic techniques provide a broad window of detection relative to direct virus detection, which is limited to the period of active infection/virus replication. Only 37 of >72 suspected or probable cases of monkeypox were confirmed by direct virologic methods during the US outbreak (www.cdc.gov/od/oc/media/mpv/cases.htm) (*6*). The US outbreak provided the first identification of human monkeypox outside Africa and showed the importance of monkeypox and other geographically limited zoonoses in an increasingly connected global community. Better diagnostics will help in measuring the effect of monkeypox in disease-endemic areas and will be important in effective outbreak detection during accidental or intentional release of virulent orthopoxviruses. We have described 2 independent methods for serologic confirmation of monkeypox infection that will be useful for these purposes.

Oregon Health and Science University (OHSU) and M.K.S. have a financial interest in Najít Technologies, Inc. (Portland, OR, USA), a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest has been reviewed and managed by OHSU and the Integrity Program Oversight Council.

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include identifying mechanisms involved with attaining long-term immunologic memory and development of more effective vaccines and diagnostics.

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Wild Ducks as Long-Distance Vectors of Highly Pathogenic Avian Influenza Virus (H5N1)

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Wild birds have been implicated in the expansion of highly pathogenic avian influenza virus (H5N1) outbreaks across Asia, the Middle East, Europe, and Africa (in addition to traditional transmission by infected poultry, contaminated equipment, and people). Such a role would require wild birds to excrete virus in the absence of debilitating disease. By experimentally infecting wild ducks, we found that tufted ducks, Eurasian pochards, and mallards excreted significantly more virus than common teals, Eurasian wigeons, and gadwalls; yet only tufted ducks and, to a lesser degree, pochards became ill or died. These findings suggest that some wild duck species, particularly mallards, can potentially be long-distance vectors of highly pathogenic avian influenza virus (H5N1) and that others, particularly tufted ducks, are more likely to act as sentinels.

The currently ongoing outbreaks caused by highly pathogenic avian influenza virus (HPAIV) of the subtype H5N1 are of concern not only to the poultry industry but also to public health (*1*,*2*). This virus, which causes a high fatality rate among infected patients, may adapt to efficient human-to-human transmission and thus initiate a new human influenza pandemic (*3*). Since 1996, when the ancestor virus was identified in domestic geese from China (*4*), outbreaks have spread and now encompass countries in Asia, the Middle East, Europe, and Africa (*5*). This spread of HPAIV among poultry flocks is traditionally thought to occur by transport of infected poultry, contaminated equipment, and persons associated with the poultry industry (*6*). HPAIV has occasionally been detected in wild birds near affected poultry flocks, but these birds have had limited or no role in virus dissemination (*7*). In the current outbreaks, however, wild birds are suspected of playing a major role as long-distance virus vectors.

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During the expansion of HPAI (H5N1) outbreaks from Asia to Europe, 2 events implicated wild birds, particularly waterbirds, as long-distance virus vectors (*8*). First, virus outbreaks in 2005 rapidly spread westward from Russia and Kazakhstan in July and August to Turkey, Romania, and Ukraine in October. Wild waterbirds were suggested as a vector because the virus spread through areas that had no record of any virus presence and coincided with the fall migration of wild waterbirds between these areas. Second, at the beginning of 2006, HPAIV (H5N1) was detected in many wild waterbirds in western Europe, often in areas where no outbreaks had been detected among intensively surveyed poultry (*9*–*12*); this event overlapped with unusual waterbird movements associated with cold weather in the Black Sea area. Quantitative analysis of the global spread of HPAIV (H5N1) also supports the potential role of migratory wild birds in virus spread (*13*). In June and July of 2007, Germany, France, and the Czech Republic again reported HPAIV (H5N1) in wild waterbirds (*14*), which illustrates their ongoing involvement in the epidemiology of this viral infection.

The main argument against the view that wild waterbirds are long-distance vectors of HPAIV (H5N1) is that most wild waterbirds in which this virus was identified were either sick or dead, which suggests that they were too severely affected to spread the virus over any substantial distance (*15*). This argument is supported by experimental evidence that over time HPAIV (H5N1) has become more pathogenic for domestic ducks. Although domestic ducks did not show clinical disease or death from HPAIV (H5N1) isolates from 2001 or before, experimental infection of domestic ducks with an HPAIV (H5N1) isolate from 2002 caused neurologic disease and death (*16*). This high pathogenicity has been shown to be associated with molecular changes in the polymerase genes PA and PB1 (*17*).

However, little is known about the pathogenicity and excretion pattern of recent HPAIV (H5N1) isolates in wild waterbird species whose migration patterns correspond with the observed westward expansion of HPAI (H5N1) outbreaks.

To test the hypothesis that wild waterbirds can excrete HPAIV (H5N1) in the absence of debilitating disease and so potentially act as long-distance virus vectors, we experimentally infected 6 species of wild ducks with an avian isolate of HPAIV (H5N1) from Europe, obtained in 2005 (A/turkey/Turkey/1/2005). We chose ducks because they are an important group in the epidemiology of avian influenza in the wild, although other waterbird species, such as geese, swans, and gulls, also play a role (*15*). We chose these particular duck species because of their abundance, preference for freshwater habitats, and migratory pattern spanning Asia, Europe, and Africa (Table; online Appendix Figure 1, available from www.cdc.gov/EID/content/14/4/600-appG1.htm). All 6 species are listed by the European Union as carrying a higher risk for avian influenza (*18*).

Materials and Methods

Virus Preparation

A virus stock of influenza virus A/turkey/Turkey/1/2005 (H5N1) was prepared by 2 passages in 10-day-old embryonated chicken eggs. The harvested allantoic fluid had a titer (*19*) of 1.3×10^8 median tissue culture infectious dose ($TCID_{50}$)/mL and was diluted with phosphate-buffered saline (PBS) to obtain a final titer of 3.3×10^3 $TCID_{50}$/mL. All experiments with HPAIV (H5N1) were performed under Biosafety Level 3+ conditions.

Animals

We experimentally infected 6 species of ducks: 2 species of diving ducks belonging to the genus *Aythya* (tufted duck [*A. fuligula*] and Eurasian pochard [*A. ferina*]) and 4 species of dabbling ducks belonging to the genus *Anas* (mallard [*A. platyrhynchos*], common teal [*A. crecca*], Eurasian wigeon [*A. penelope*], and gadwall [*A. strepera*]). The *Anas* species represent 3 clades: the mallard represents the mallard clade, the common teal represents the green-winged teal clade, and the Eurasian wigeon and gadwall represent the wigeon clade, previously belonging to the genus *Strepera* (*20*). For each species, males and females were equally represented. All ducks used for the infection experiments were captive-bred (Dierenhandel Hoogendoorn, Stolwijk, the Netherlands) and housed indoors since hatching to minimize the risk for inadvertent avian influenza virus infection. Birds were 8–11 months of age at time of inoculation. Serum samples, cloacal swabs, and pharyngeal swabs were collected from all ducks 1 week before inoculation. Serum was analyzed by using a commercially available influenza A virus antibody ELISA kit for the detection of antibodies against nucleoprotein (European Veterinary Laboratory, Woerden, the Netherlands) according to the manufacturer's instructions. Swabs were tested by reverse transcription–PCR (RT-PCR). No duck had antinucleoprotein antibody, except 1 pochard. Its serologic status did not protect it from HPAIV (H5N1) infection; it had the most severe clinical signs of all inoculated pochards and died at 4 days postinoculation (dpi). For 1 teal and 2 pochards, titers were suspected positive. No duck used for the infection experiments was positive for avian influenza virus by RT-PCR. We used 8 specific-pathogen–free White Leghorn chickens, 4–6 weeks old, as controls for the pathogenicity of the virus stock.

Experimental Design

For each species, 8 birds were housed together in negatively pressurized isolator units. Each bird was inoculated with 1×10^4 $TCID_{50}$ HPAIV (H5N1), 1.5 mL intratracheally and 1.5 mL intraesophageally. We used this low dose to increase the chance of inducing a subclinical infection and to simulate field circumstances. In addition, 4 birds per duck species, which served as negative controls, were sham inoculated in the same manner with PBS-diluted sterile allantoic fluid. Each day, a qualified veterinarian scored clinical signs of disease in all birds according to a standardized list. Cloacal and pharyngeal swabs were collected in 1 mL transport medium (*21*) daily for the first 14 days and every 2 days thereafter.

Table. Health status and virus excretion of 46 wild ducks experimentally infected with highly pathogenic avian influenza virus (H5N1)

Common name (taxonomic name), n = 8 each	No. ducks with clinical signs		No. ducks that excreted virus from*			
			Pharynx		Cloaca	
	Mild	Severe	Virus isolation	RT-PCR	Virus isolation	RT-PCR
Tufted duck (*Aythya fuligula*)†	4	3	6	7	0	5
Eurasian pochard (*Aythya ferina*)†	3	1	7	7	2	5
Mallard (*Anas platyrhynchos*)	0	0	8	8	0	5
Common teal (*Anas crecca*)	0	0	3	7	1	4
Eurasian wigeon (*Anas penelope*)	0	0	4	7	0	0
Gadwall (*Anas strepera*)	0	0	7	8	0	8
Total	7	4	35	44	3	27

*Positive result from any swab collected during the experiment. RT-PCR, reverse transcription–PCR.
†One bird removed after inoculation because of concurrent disease.

We randomly divided each group of 8 birds into 2 groups of 4. One group was euthanized by exsanguination under isoflurane anesthesia for pathologic examination at 4 dpi; the other group was monitored for virus excretion until 18–21 dpi. Two ducks were removed after inoculation: 1 tufted duck because of concurrent aspergillosis and 1 pochard because of concurrent staphylococcosis. Also, 1 pochard and 3 tufted ducks were were chosen for pathologic examination at 4 dpi because they were dead or moribund. Although this was not random sampling, it does reflect the field situation because dead ducks no longer actively excrete virus. As expected, by 2 dpi 100% of the positive-control chickens were sick or dead, whereas the negative-control ducks showed no clinical signs and were euthanized at 4 dpi. Animal studies were approved by an independent animal ethics committee and performed under Biosafety Level 3+ conditions.

Pathologic Examination and Immunohistochemical Testing

Necropsies and tissue sampling were performed according to a standard protocol. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were examined by 1 of 2 methods: hematoxylin and eosin staining for histologic evaluation or an immunohistologic method that used a monoclonal antibody against nucleoprotein of influenza A virus as a primary antibody for detection of influenza viral antigen (*22*). The positive control was lung tissue of an HPAIV (H5N1)–infected domestic cat; negative controls were omission of primary antibody, substitution of primary antibody by an irrelevant monoclonal antibody of the same isotype, and testing of tissues from sham-inoculated ducks. The following tissues were examined: brain (cerebrum, cerebellum, brainstem), trachea, bronchus, lung, caudothoracic or abdominal air sac, esophagus, proventriculus, duodenum, pancreas, liver, jejunum, ileum, cecum, colon, bursa of Fabricius, spleen, kidney, gonad (testis or ovary), heart, pectoral muscle, and adrenal gland.

RT-PCR and Virus Titration

Tissue samples were weighed and homogenized in 3 mL of transport medium by use of a homogenizer (Kinematica Polytron, Lucerne, Switzerland). RNA isolation and RT-PCR were performed as described (*23*). Briefly, RNA from swabs and tissue suspensions was isolated by using a MagNaPure LC system with the MagNaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Almere, the Netherlands). Real-time RT-PCR assays were performed on an ABI Prism 7000 Sequence Detection System machine (Applied Biosystems, Foster City, CA, USA) by using the TaqMan EZ RT-PCR Core Reagents Kit (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) according to the manufacturer's instructions. The test used a hybridization probe (5′-6-FAM-TTT-ATT-CAA-CAG-TGG-CGA-GTT-CCC-TAG-CAC-T-TAMRA-3′) and specified primers (forward: 5′-GAG-AGG-AAA-TAA-GTG-GAG-TAA-AAT-TGG-A-3′ and reverse: 5′-AAG-ATA-GAC-CAG-CTA-CCA-TGA-TTG-C-3′) to detect the hemagglutinin gene of HPAIV (H5N1). For each run the samples were prepared and processed in parallel with several negative and positive control samples. Virus titers were determined by serial 10-fold dilution of the homogenized tissue samples and swabs on MDCK cells, as described (*19*). Virus titrations were performed in quadruplicate.

Results

Despite the low doses of virus used to inoculate the ducks, rates of productive infection in the 6 species were high: 76% according to virus isolation and 93% according to RT-PCR (Table). HPAIV (H5N1) infection caused clinical signs of disease in only tufted ducks and pochards, both of which are diving ducks in the genus *Aythya* (Table). In contrast, the remaining 4 species—all dabbling ducks belonging to the genus *Anas*—were clinically unaffected. Clinical signs, which were more severe in tufted ducks than in pochards, developed at 3 to 4 dpi and consisted of labored breathing, increased recumbency, and neurologic signs (torticollis [Figure 1, panel A], circling, loss of balance, and head tremors). Severely affected birds died or were euthanized in a moribund state at 4 dpi. Mildly affected birds recovered by 7 or 8 dpi.

Severe neurologic signs in tufted ducks and pochards were associated with multifocal viral encephalitis. Although no gross brain lesions were noted (online Appendix Table 1, available from www.cdc.gov/EID/content/14/4/600-appT1.htm), according to laboratory analysis these birds had multiple foci of influenza virus antigen expression (Figure 1, panel B; online Appendix Table 2, available from www.cdc.gov/EID/content/14/4/600-appT2.htm) associated with severe necrosis and inflammation (Figure 1, panel C) and high virus titers ($10^{3.5}$ to $10^{5.2}$ $TCID_{50}$ per g tissue) in the brain (online Appendix Table 3, available from www.cdc.gov/EID/content/14/4/600-appT3.htm). The only other ducks with evidence of HPAIV (H5N1) infection of the brain were gadwalls, none of which showed neurologic signs. Gadwalls had only focal influenza virus antigen expression (Figure 1, panel D; online Appendix Table 2), mild encephalitis (Figure 1, panel E), and low virus titers ($10^{1.5}$ $TCID_{50}$ per g tissue) in the brain (online Appendix Table 3). No other species had evidence of HPAIV (H5N1) infection in the brain according to immunohistochemical testing, histologic examination, or virus isolation (Figure 1, panels F and G; online Appendix Tables 2 and 3), although

individual animals did have positive RT-PCR results for the brain (online Appendix Table 4, available from www.cdc.gov/EID/content/14/4/600-appT4.htm).

Pharyngeal excretion of HPAIV (H5N1) varied significantly among the 6 duck species (1-way analysis of variance of area under pharyngeal excretion curve up to 4 dpi, $p<0.001$), by virus isolation (Figure 2, panel A) and by RT-PCR (Figure 2, panel C). The ducks could be divided into a high-excretion group consisting of tufted ducks, pochards, and mallards, and a low-excretion group consisting of teals, wigeons, and gadwalls (Figure 2, panels B and D). Pharyngeal excretion also varied substantially among individuals within species (online Appendix Figures 2 and 3, available from www.cdc.gov/EID/content/14/4/600-appG2.htm and www.cdc.gov/EID/content/14/4/600-appG3.htm, respectively.). This finding was most extreme in tufted ducks and pochards, the species in which the individuals with the highest excretion level were also those showing severe clinical signs (Figure 2, panels B and D).

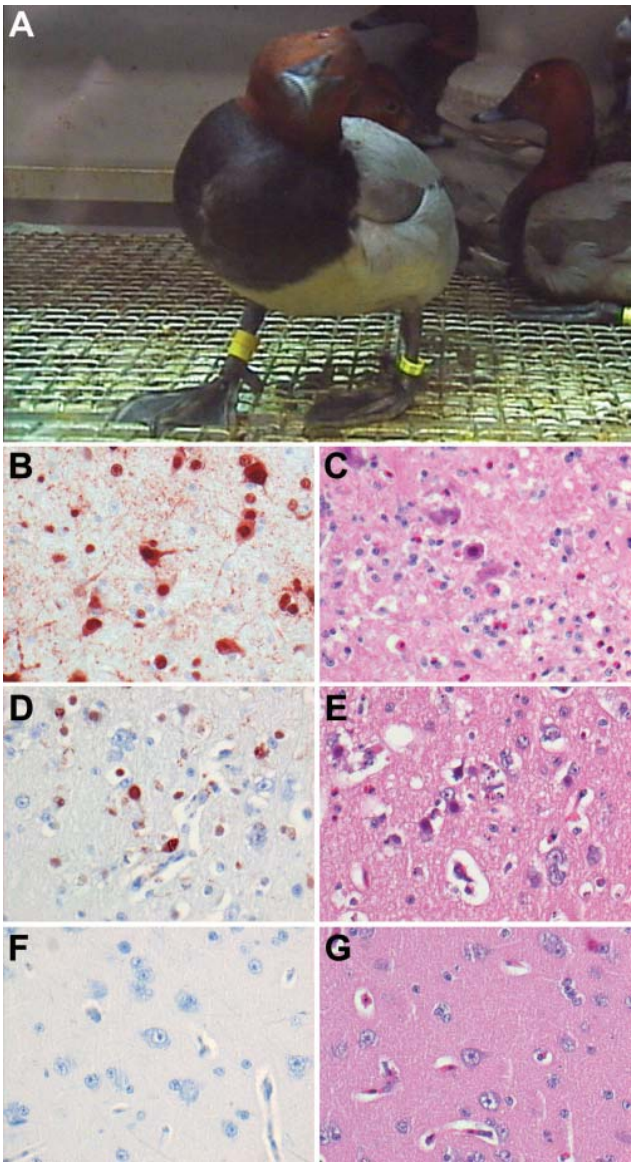

Figure 1. Central nervous system changes in wild ducks experimentally infected with highly pathogenic avian influenza virus (H5N1). A) Torticollis in a pochard. B) Severe multifocal encephalitis, characterized by abundant influenza virus antigen expression in neurons and glial cells and C) extensive necrosis and inflammation, in a tufted duck. D) Rare virus antigen expression in neurons and E) mild necrosis and inflammation in a gadwall that did not show neurologic signs and had only mild focal encephalitis. F) Lack of virus antigen expression and G) lack of necrosis and inflammation in brain tissue of a mallard that did not show neurologic signs. Tissues were stained either by immunohistochemistry that used a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody (B, D, F) or with hematoxylin and eosin (C, E, G); original magnification ×100.

Pharyngeally excreted HPAIV (H5N1) likely originated from lung, air sac, or both, because these were the only tissues in the respiratory tract that had immunohistochemical evidence of virus replication (Figure 2, panels E and G; online Appendix Table 2) and because virus was frequently detected in these tissues by virus isolation (online Appendix Table 3) and RT-PCR (online Appendix Table 4). The histologic lesions corresponding to influenza virus antigen expression in these tissues were bronchointerstitial pneumonia (Figure 2, panel F) and lymphocytic airsacculitis (Figure 2, panel H). Despite frequent isolation of HPAIV (H5N1) from trachea and extrapulmonary bronchus (online Appendix Table 3), these tissues had neither histopathologic nor immunohistochemical evidence of HPAIV (H5N1) replication (online Appendix Table 2), which suggests that virus isolated from these sites at 4 dpi originated from elsewhere in the respiratory tract.

Cloacal excretion of HPAIV (H5N1) was uncommon; virus was detected in cloacal swabs of only 7% of ducks by virus isolation and 59% by RT-PCR (Table). Cloacal excretion was exceeded by pharyngeal excretion in all 6 duck species, according to virus isolation (Figure 3, panels A and B; online Appendix Figure 2) and RT-PCR (Figure 3, panels C and D; online Appendix Figure 3).

Cloacally excreted virus likely originated from pancreas, liver, or both, on the basis of the significant association between virus antigen expression in these tissues (online Appendix Table 2) and virus detection in cloacal swabs by RT-PCR (online Appendix Table 4); 7 of 8 birds with virus antigen expression in liver, pancreas, or both had PCR-positive cloacal swabs between 1 and 4 dpi, in contrast to 0 of 15 birds without virus antigen expression in these tissues (Fisher exact test, $p<0.00001$). In the pancreas, tufted ducks and pochards had multifocal necrosis (Figure 3, panel E), which was the most prominent gross lesion associated with HPAIV (H5N1) infection in this study (online Appendix Table 1). Virus antigen expression (Figure 3, panel F) occurred at the transition between normal parenchyma and these necrotic foci (Figure 3, panel G) and

corresponded with high virus titers ($10^{1.5}$ to $10^{6.2}$ $TCID_{50}$ per g tissue) in the pancreas (online Appendix Table 3). In the liver, widespread virus antigen expression (Figure 3, panel H) was associated with necrotizing hepatitis (Figure 3, panel I) and variable virus titers (no virus isolated to $10^{6.2}$ $TCID_{50}$ per g tissue) in the liver (online Appendix Table 3). Virus produced in pancreas and liver could potentially have reached the intestinal lumen through pancreatic and bile ducts, respectively. Although virus antigen expression was detected in several other tissues, virus originating from these sites likely did not contribute to virus excretion.

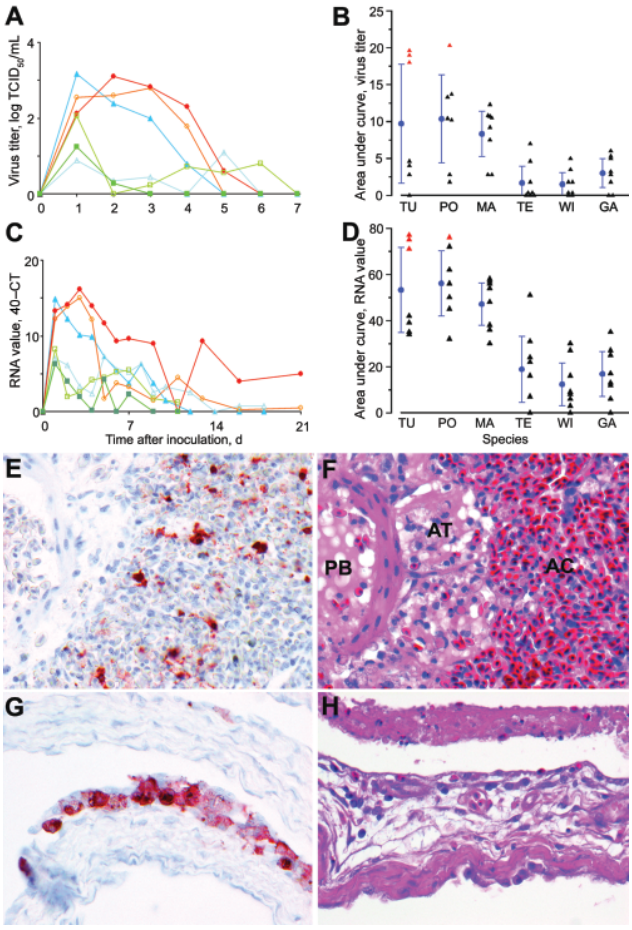

Figure 2. Mean pharyngeal excretion of highly pathogenic avian influenza virus (H5N1) of wild ducks by A) virus isolation and C) reverse transcription–PCR (RT-PCR). Pochard (red, closed circle), tufted duck (orange, open circle), mallard (dark blue, closed triangle), teal (light blue, open triangle), wigeon (dark green, closed square), gadwall (light green, open square); $TCID_{50}$, median tissue culture infectious dose; Ct, cycle threshold. Area under the curve in the first 4 days postinoculation (mean ± 95% confidence interval) for B) virus isolation and D) RT-PCR. TU, tufted duck; PO, pochard; MA, mallard; TE, teal; WI, wigeon; GA, gadwall; red triangles, birds with severe clinical signs; black triangles, birds with mild or no clinical signs. E) Influenza virus antigen expression in epithelial cells in bronchus, parabronchus, atrium, and air capillaries of a tufted duck. F) Bronchointerstitial pneumonia, characterized by flooding of parabronchi (PB), atria (AT), and air capillaries (AC) with proteinaceous fluid and inflammatory cells in a tufted duck. G) Influenza virus antigen expression in epithelial cells lining the air sac wall and H) epithelial necrosis and lymphocytic infiltration in a gadwall. E–H original magnification ×100. Tissues were stained either by immunohistochemistry that used a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody (E, G) or with hematoxylin and eosin (F, H).

It is unlikely that cloacally excreted virus originated from the intestinal, urinary, or genital tracts, although all 3 tracts empty into the cloaca. In the intestine, no virus antigen expression was found in the intestinal epithelium of any of the 23 ducks examined. Virus antigen expression was found in neurons and satellite cells in the peripheral nervous system (submucosal and myenteric plexi, mesenteric ganglia) of the small intestine (online Appendix Figure 4, panel A, available from www.cdc.gov/EID/content/14/4/600-appG4.htm), in association with necrotizing ganglioneuritis (online Appendix Figure 4, panel B), and in myocytes in the lamina muscularis mucosae of the colon, without associated histologic lesions. However, these tissues do not empty into the intestinal lumen. No virus antigen expression was found in tissues of urinary tract (kidney) or genital tract (testis or ovary) (online Appendix Table 2). The occasional isolation of HPAIV (H5N1) from kidney samples (online Appendix Table 3) may be explained by inadvertent sampling of overlying air sac wall, which did express influenza virus antigen.

Evidence of HPAIV (H5N1) replication was found sporadically in tissues other than those of the respiratory, digestive, and nervous systems (online Appendix Table 2). Virus antigen expression was detected in multiple foci of medullary and cortical cells of the adrenal gland (online Appendix Figure 4, panel C) and was associated with necrotizing adrenalitis (online Appendix Figure 4, panel D). Virus antigen expression was also detected in multiple foci of myocytes of the heart and was associated with myocardial necrosis.

Discussion

Our study shows that of the 6 wild duck species studied, the mallard is the prime candidate for being a long-distance vector of HPAIV (H5N1) because it was the only species to show abundant virus excretion without clinical or pathologic evidence of debilitating disease (Table; Figure 2, panels B and D). These findings fit with the absence of dead mallards in wild bird die-offs from HPAIV (H5N1) in Europe and Asia in 2005 and 2006 (*14*,*24*,*25*), although HPAIV (H5N1) was detected in 1 dead mallard during the recent 2007 HPAI (H5N1) outbreak in wild birds in Germany (*26*). Other characteristics of the mallard support its potential role as a vector ([*27*]*;* online Appendix Figure 1): it is the most abundant anatid species in Western Eurasia (≈9 million birds); part of the population migrates long distances northeast to southwest between breeding and winter-

ing areas; and it is found on nearly every type of wetland and is very tolerant of human presence, thus forming a potential link between wild waterfowl, domestic animals, and humans.

Pochards and tufted ducks are less likely candidates as long-distance virus vectors because those individuals that excreted the most virus also developed severe neurologic disease (Figure 2, panels B and D) and therefore would not have been able to fly far before succumbing. Instead, they are more likely to act as sentinels for HPAIV (H5N1) in wild bird populations, as do mute swans (*Cygnus olor*) (*9*). However, pochards cannot be ruled out as potential vectors because some birds excreted abundant virus in absence of severe clinical signs (Figure 2, panels B and D). Our results correspond with field observations of pochards and tufted ducks involved in wild bird die-offs from HPAIV (H5N1) infection in France, Germany, and Sweden early in 2006 (*14*). Some of these birds showed clinical signs of neurologic disease, e.g., compulsively swimming around in circles (*28*). Therefore, close surveillance of these 2 *Aythya* species for unusual illness, particularly neurologic disease, or death should provide early warning for HPAIV (H5N1) infection in an area. Redheads (*Aythya americana*), which are diving ducks indigenous to North America, experimentally infected with a 2005 isolate of HPAIV (H5N1) neither showed clinical signs nor died (*29*). Of the 6 species tested, the 3 remaining *Anas* species—gadwall, teal, and wigeon—are the least likely candidates as long-distance virus vectors because they had limited virus excretion (Figure 2, panels B and D).

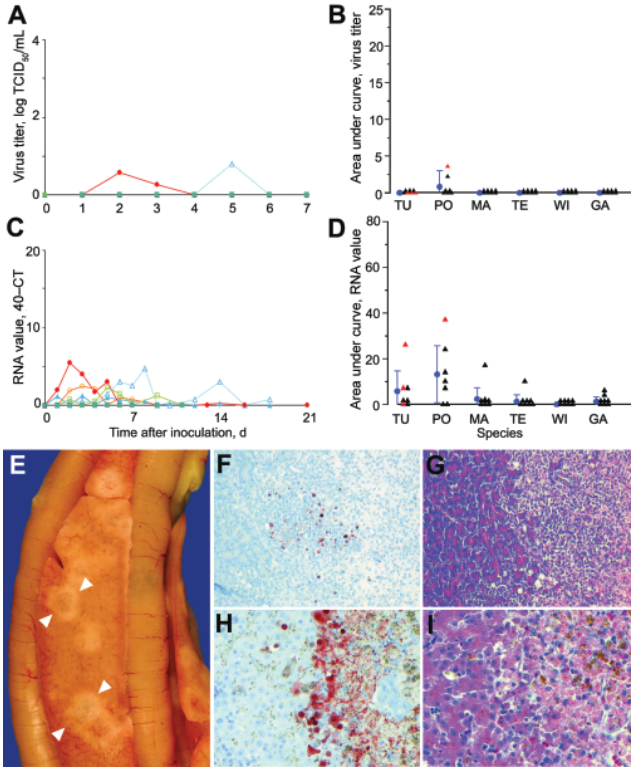

Figure 3. Mean cloacal excretion of highly pathogenic avian influenza virus (H5N1) by wild ducks by A) virus isolation and C) reverse transcription–PCR (RT-PCR). Legend for panels A–D as in Figure 2. E) Pancreas showing multiple foci of necrosis (between arrowheads) in a pochard. F) Pancreatic acinar cells in a pochard and H) hepatocytes in a tufted duck, showing the transition area between normal and necrotic tissue expressing abundant influenza virus antigen. G) Pancreatic lesions in a pochard and I) hepatic lesions in a tufted duck, characterized by sharp transition between normal tissue (left side of panels) and foci of necrosis and inflammatory cell infiltration (right side of panels). F, G original magnification ×50. H, I original magnification ×100. Tissues were stained either by immunohistochemistry that used a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody (F, H) or with hematoxylin and eosin (G, I).

HPAIV (H5N1) infection in these wild ducks contrasts in pattern of excretion with that of low-pathogenicity avian influenza virus infection in wild ducks and contrasts in pattern of disease with that of HPAIV infection in chickens. Both contrasts can be explained by the specific tissue tropism of HPAIV (H5N1) in wild ducks. With regard to pattern of excretion, low cloacal excretion was associated with lack of evidence for HPAIV (H5N1) replication in intestinal epithelium of any of the 23 ducks examined (online Appendix Table 2), in contrast to most low-pathogenicity avian influenza viruses for which intestine is the main replication site (*30*). Instead, HPAIV (H5N1) replicated preferentially in the respiratory tract (online Appendix Tables 2 and 4), which corresponds with high pharyngeal excretion. How this preferential pharyngeal excretion of HPAIV (H5N1) affects its spread and persistence in a wild duck population remains to be determined.

Severe clinical disease in the HPAIV (H5N1)–infected tufted ducks and pochards manifested itself mainly as neurologic signs at about 4 dpi, although pathologic examination also showed virus-induced lesions in organs other than the brain. These findings differ substantially from those of HPAIV (H5N1)–infected chickens, which are characterized mainly by widespread hemorrhage and edema and death by about 2 dpi (*31*). Again, this contrast can be explained by differences in tissue tropism. Whereas the cardiovascular lesions in poultry are associated with widespread replication of HPAIV (H5N1) in endothelium lining the blood vessels (*31*), no such endotheliotropism was detected in any of 23 ducks examined.

The knowledge gained from this study has several implications for surveillance in wild ducks. Active surveillance (sampling of apparently healthy wild birds) should give priority to mallards and, to a lesser degree, pochards. Sampling should not be limited to cloacal swabs, as is the custom in surveillance for low-pathogenicity avian influenza virus, but should include pharyngeal swabs. Passive surveillance (sampling of diseased or dead birds), should pay extra attention to tufted ducks and pochards, particu-

larly those exhibiting neurologic disease. Sampling of wild duck carcasses should not be limited to cloacal, pharyngeal, and tracheal swabs and should include internal organs such as brain, trachea, lung, pancreas, liver, kidney, and spleen (online Appendix Tables 3 and 4).

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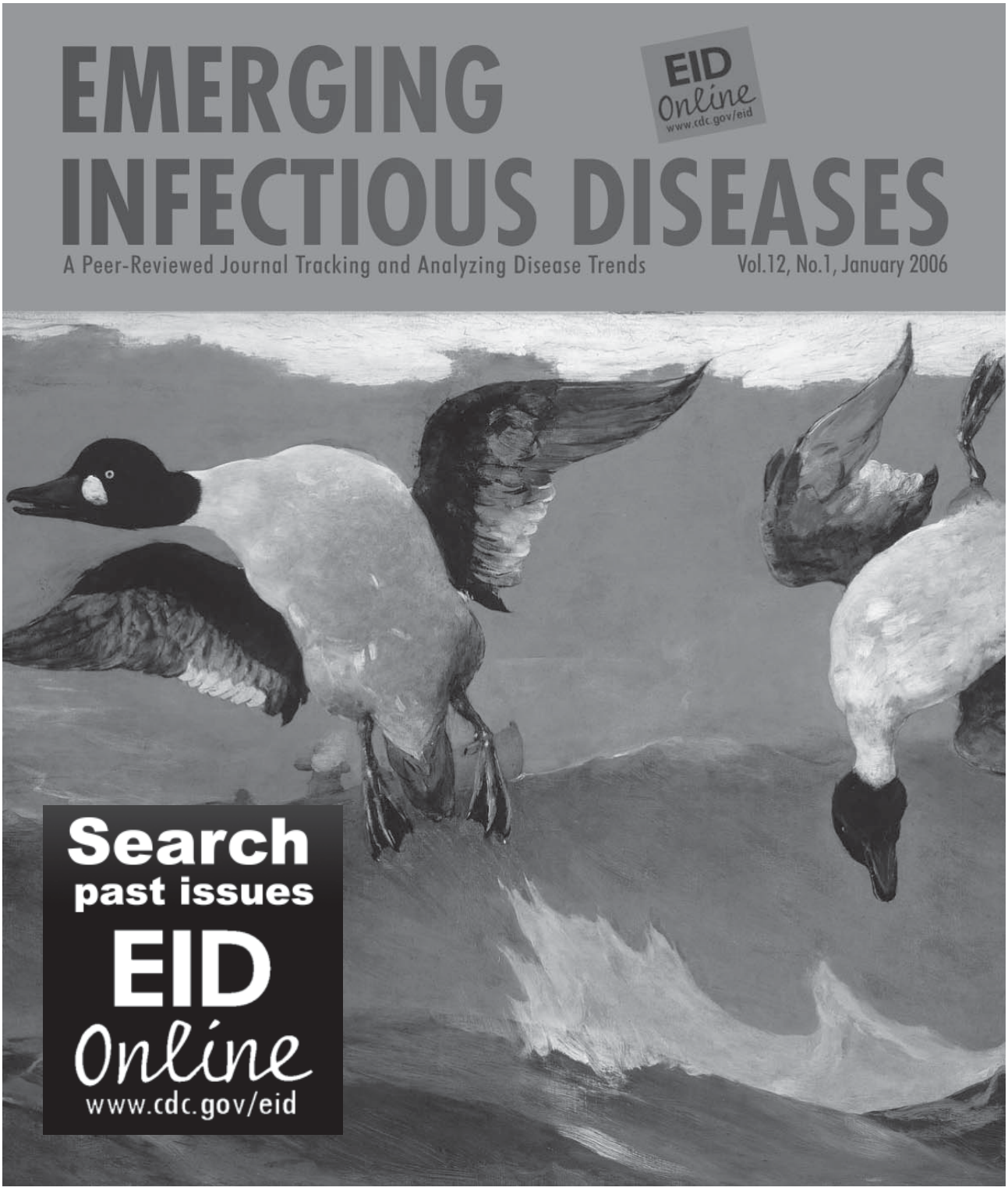

Rapid Typing of Transmissible Spongiform Encephalopathy Strains with Differential ELISA

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The bovine spongiform encephalopathy (BSE) agent has been transmitted to humans, leading to variant Creutzfeldt-Jakob disease. Sheep and goats can be experimentally infected by BSE and have been potentially exposed to natural BSE; however, whether BSE can be transmitted to small ruminants is not known. Based on the particular biochemical properties of the abnormal prion protein (PrPsc) associated with BSE, and particularly the increased degradation induced by proteinase K in the N terminal part of PrPsc, we have developed a rapid ELISA designed to distinguish BSE from other scrapie strains. This assay clearly discriminates experimental ovine BSE from other scrapie strains and was used to screen 260 transmissible spongiform encephalopathy (TSE)–infected small ruminant samples identified by the French active surveillance network (2002/2003). In this context, this test has helped to identify the first case of natural BSE in a goat and can be used to classify TSE isolates based on the proteinase K sensitivity of PrPsc.

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases affecting humans and animals; examples are scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease in cervids. Strong evidence indicates that the BSE agent has been transmitted to humans through the food chain, leading to the emergence of variant Creutzfeldt-Jakob disease (vCJD). Recently, the first case of natural BSE was identified in a French goat (*1*), and a second probable one was reported in the United Kingdom (*2*). It seems, however, that these were isolated cases and that the BSE level in small ruminant flocks is probably low. However, BSE can be transmitted experimentally to sheep and goats by the oral route (*3–5*), whereas small ruminants have very likely been exposed to the BSE agent through contaminated meat and bone meal (MBM). Given the widespread tissue distribution of the BSE agent in sheep and goats, horizontal and vertical transmission within sheep and goat populations is possible, which could lead to human exposure to the agent through the food chain, even after the MBM feed ban.

TSEs are characterized by the accumulation in the brain of an abnormal form of the prion protein (PrPsc), which is derived from the normal one (PrPc) and manifests as unusual biochemical properties including insolubility in the presence of detergents and partial resistance to the action of proteases like proteinase K (PK). The conventional method of identifying a TSE strain is the experimental transmission of the disease in several mouse lines (*6*,*7*) including transgenic mouse lines that over express PrP, thereby increasing the efficiency and speed of transmission (*8–10*). However, these methods cannot be used as screening methods.

Sophisticated histopathologic and immunohistochemical methods have also been used to identify TSE strains (*5*,*11–15*). These methods appear efficient in differentiating BSE from other scrapie strains, but they are time-consuming and not suitable for large-scale typing.

Several studies have shown that the resistance of PrPsc to PK treatment varies depending on the strain (*16–18*). PrPsc associated with BSE in cattle or sheep is more sen-

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sitive to PK degradation at its amino-terminus than PrPsc from scrapie. Recently "atypical" forms of scrapie sensitive to PK, such as the Nor98 strain, have been described (*19*). Since 2002, large-scale epidemiologic studies have led to the identification of hundreds of atypical cases in several European countries (*20*); these cases included an unusual occurrence in sheep with genotypes previously associated with high resistance to scrapie (including the ARR/ARR genotype) (*21*). Converging data show that most atypical cases share identical biochemical and biologic features with Nor-98 (*22*).

Glycoform ratios and the molecular migration pattern of PK-treated PrPsc, as obtained in Western blot techniques, enable characterization of scrapie strains and BSE. (These topics have been reviewed by Groschup et al. [*23*]) This method identified similarities between BSE in cattle and experimental BSE in sheep (*24*,*25*) and similarities between vCJD and BSE in cattle (*26*,*27*). However, these typing immunoblotting techniques are not easily applied to test huge numbers of field samples. To increase specificity, a differential immunoblotting technique involving 2 specific antibodies, directed against the amino- and carboxy-termini of PrP, has been developed (*10*,*28*). Comparison of the signals obtained for both antibodies provides an easy and fast identification of the BSE strain because binding of the anti-N terminal antibody is almost completely suppressed. This approach has been used to characterize unusual BSE cases in cattle (*29*) and naturally infected scrapie sheep (*14*,*30*).

We describe an ELISA designed to distinguish BSE and scrapie strains, on the basis of their differential resistance to PK. The design of this method is similar to that of a rapid test widely used in Europe (TeSeE, Bio-Rad, Hercules, CA, USA) and allows rapid screening of a large number of sheep samples previously shown to contain PK-resistant prion protein (PrPres) by a conventional test.

Experimental Procedures

Materials

Chemicals were obtained from Sigma (St. Louis, MO, USA) except (aminoethyl)benzenesulfonyl fluoride) (AEBSF) from ICN Pharmaceuticals, Inc. (Costa Mes, CA, USA). All other reagents for purification and detection of PrPres were from the Bio-Rad TeSeE and TeSeE Sheep & Goat kits.

Samples

Experimental Samples

Experimental samples for scrapie controls 99–1316 and 99–1487 were from the Veterinary School of Toulouse (France) and for PG1259 from the Veterinary Laboratories Agency (VLA, Weybridge UK). Experimental ovine BSE-infected samples (SB1 and SB3 [*31*]) were from Agence Française de Sécurité Sanitaire des Aliments (AFSSA), the French national reference laboratory in Lyon. Some of the first-passage experimental ovine BSE-infected animals (at the clinical stage of the disease) were produced by UR INRA 1282 Nouzilly (intracerebral inoculation) in the framework of the European "BSE in sheep" QLKCT 2001–01309 program (nos. 347, 359, 378, 384, 386, 388, 397, bearing ARQ/ARQ genotype; nos. 12, 38, 331, 337, 341, 368, and 369 had the ARR/ARR genotype). First-passage experimental ovine BSE-infected animals 7704, 7705, PG0637/01, PG0638/01, PG0639/01, and PG0640/01 as well as second-passage animals 1630M, 1631M, 1636M, 1637M, 1638M, 1639M, 1641M, 1643M, 1644M, and 1645M were from VLA. Experimental caprine BSE samples were from the Institute for Animal Health, Edinburgh, Scotland. Nor98 isolates were from the National Veterinary Institute, Oslo, Norway.

Field Isolates

This study tested, in blind conditions, 270 samples provided by AFSSA. This represents all the TSE-positive cases recorded during 2002–2003 by active surveillance (>160,000 brain samples originating from slaughterhouse and rendering plants screened by rapid tests) and is thus representative of the diversity of French isolates. The 270 samples included 21 caprine isolates and 42 cases of atypical scrapie previously identified by AFSSA.

Homogenization of Nervous Tissues

Nervous tissues were homogenized and calibrated according to the Bio-Rad purification protocol. Homogenates (20% w/v) were diluted 10-fold in a negative sheep brain homogenate or tested undiluted if absorbance measurement in A reagent was <0.5.

Processing of Samples (Scrapie-associated Fibrils Preparation)

Two batches (A and A′ series) of 21 samples (250 μL (20% homogenate) of each sample per tube, manual procedure) or 29 samples (250 μL of each sample per well in 2 deep-well plates, automated protocol) were analyzed together with 2 scrapie controls (nerve tissue from 2 scrapie sheep, 99–1316 and PG1259 [manual] or 99–1316 and 99–1487 [automated]) and an experimental BSE-infected sheep (397BS, INRA). Each batch was treated in 1 set of conditions of PK treatment. For the manual protocol, 250 μL of A reagent (TeSeE purification kit) containing PK (10 μg) is distributed in each tube of the A series, and 250 μL of A′ reagent/PK (5% N-lauroylsarcosine sodium salt [w/v], 5% [w/v] sodium dodecyl sulfate [SDS] containing 27.5 μg of PK) in each tube of the A′ series. All the tubes were then

homogenized by 10 inversions and incubated at 37°C for 15 min. Then, 250 µL of B reagent (Bio-Rad purification kit)/phenylmethylsulfonyl fluoride (PMSF) (final concentration 4 mmol/L) was added, before homogenization and centrifugation for 5 min at 20,000 × *g* at 20°C.

For the automated protocol, the deep-well plates were successively incubated for 12 min at 37°C in the TeSeE NSP automated system. Each well of the first plate was processed with 250 µL of A reagent/PK before 15 min of incubation at 37°C. Then 250 µL of the B reagent containing PMSF (12 mmol/L) was added and incubated 5 min at 37°C. The deep-well plate was centrifuged for 10 min at 2,000× *g*, 4°C. The second plate (A′ series) was processed similarly with the A′ reagent/PK.

For both protocols, supernatants were discarded and the tubes (or the plates) dried by inversion on absorbent paper for 5 min. Each pellet was denatured for 5 min at 100°C with 25 µL of C reagent (Bio-Rad purification kit). We then added 350 µL of R6 buffer containing 4 mmol/L AEBSF. For the field isolates, serial dilutions (3- and 10-fold in R6 buffer/AEBSF) were performed to ensure an optimal ELISA signal.

Immunometric Assay

All the reagents were provided by the Bio-Rad TeSeE Sheep & Goat detection kit. We distributed, in duplicate, 100 µL of samples (undiluted, 3- and 10-fold diluted) and controls in microtiter plates coated with the first anti-PrP antibody. The plate reacted for 2 h at room temperature (RT). After 3 washing cycles (R2 buffer), 100 µL/well of the enzyme conjugate was added for 2-h reaction at RT. After 3 washing cycles, 100 µL of substrate was added for 30 min in the dark at RT, before blocking the reaction with 100 µL of stopping solution and reading the absorbance at 450/620 nm. The mean ratio of the absorbances obtained in the 2 conditions (A and A′) was calculated for each sample by selecting a range of appropriate dilutions providing absorbance measurements ranging from 0.5 to 2.5 for A reagent.

Immunoblot

Pellets of purified PrPres (TeSeE purification kit) were denatured in Laemmli buffer for 5 min at 100°C. After SDS–12% PAGE, samples were blotted on polyvinylidene difluoride membranes (Bio-Rad) and blocked with 5% bovine serum albumin. PrP was detected by using horseradish peroxidase–labeled SHa31 antibody and chemiluminescence (ECL plus Western blotting detection system, Amersham Biosciences, Piscataway, NJ, USA).

PK Range for Nor98 Isolates

Five Nor98 isolates as well as the 2 scrapie controls and the experimental ovine BSE were diluted in ovine negative brain homogenate (200- to 800-fold). In 6 sets of PK conditions 250 µL of each sample was treated: 1 with 250 µL of A TeSeE reagent (TeSeE purification kit) with 10 µg of PK and 5 different PK concentrations (10 µg, 15 µg, 20 µg, 25 µg, and 30 µg) in 250 µL of A TeSeE Sheep & Goat reagent, named A" reagent (TeSeE Sheep & Goat purification kit).

Results

Principle of the Typing Test

The typing ELISA is based on the screening test for the postmortem diagnosis of BSE initially developed by the Commissariat à l'Energie Atomique (CEA) (*32*,*33*). After selective purification of PrPres, the denatured PrPres is measured by using a sandwich assay in microtiter plates.

The capture antibody recognizes an amino terminal epitope, while the tracer antibody binds to the core of the protein (Figure 1, panel A). In the first set of conditions, the PK digestion is performed in a control medium (mixture of detergents and chaotropic agents) to preserve the N-terminal epitope (Figure 1, panels A and B, PK treatment in A conditions). By varying the conditions of PK treatment,

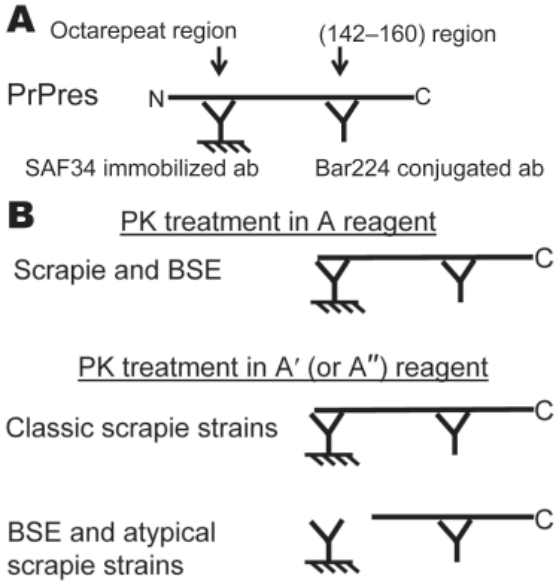

Figure 1. Principle of the 2-site immunometric typing assay. A) EIA screening test. In these conditions (Bio-Rad, Hercules, CA, USA), after mild proteinase K (PK) digestion, denatured PK-resistant prion protein (PrPres) is captured by the solid phase–immobilized antibody SAF34, recognizing the octarepeat region, and shown by the Bar224 tracer antibody, directed against the core of the protein. B) EIA typing test. In this test, the PrPres-containing sample is treated in 2 sets of PK conditions. In the first set of conditions (PK treatment in A reagent, Bio-Rad screening condition), the octarepeat region is maintained in scrapie- and bovine spongiform encephalopathy (BSE)–associated PrPres; in the second set of conditions (high PK concentration in A′ reagent), BSE- and labile strain–associated PrPres is more sensitive to PK digestion than the classic PrPres associated with scrapie strains. Calculation of the ratio in the 2 conditions differentiates BSE and labile strains from classic scrapie strains. ab, antibody.

PrPsc associated with the BSE strain appears more sensitive to PK than most of the other prion strains. Conditions of PK treatment can thus be defined for selectively deleting the epitope recognized by the capture antibody in BSE strain while preserving it in most scrapie strains (Figure 1, panel B, PK in A′ conditions), in agreement with previous reports (*5*,*10*,*23*,*28*).

The first step of the Bio-Rad test (purification and concentration of PrPres) was performed either manually or by using the NSP automated system and adapted for the typing test. Determination of the ratio between the 2 measurements (R = A/A′) allows differential detection of the BSE strain. In the current conditions, this ratio is <2 for most of the scrapie strains (manual or automated protocol) and close to 6 and 10 for the automated and manual protocols, respectively, with the BSE strain (Table 1). During the current study, we made 2 major changes with regards to the Bio-Rad test: 1) we added PK inhibitors (PMSF and AEBSF) at 2 stages of the purification procedure for better control of PK digestion, and 2) we performed a series of dilutions to determine the optimal range of absorbance, which allowed a precise determination of the A/A′ ratio (ranging between 0.5 and 2.5 absorbance unit, Figure 2, panels A and B). To minimize the interassay variations, the ratio obtained for each sample was further normalized by dividing it by the ratio obtained for the experimental ovine BSE control (Figure 2, panel C)

To evaluate this typing test, 37 brain samples from 25 ARQ/ARQ sheep experimentally infected with BSE (first or second passage) were compared with the brain samples of 3 controls. All samples analyzed in 3 independent experiments provide A/A′ ratios >7 with a mean of 11 0.2 ± 1.4 (online Appendix Figure 1, panel A, available from www.cdc.gov/EID/content/14/4/608-appG1.htm). To further investigate the possible influence of the genotype, we tested 7 spinal cord samples from experimental BSE bearing the ARR/ARR genotype, in at least 4 independent experiments. A/A′ ratios ranged from 5.5 to 6.9 (mean 6.0 ± 0.4) and were statistically different from the experimental ARQ/ARQ ovine BSE used as control (mean 8.8 ± 0.9, $p<0.001$ for all samples except no. 38, $p<0.05$) (online Appendix Figure 1, panel B).

Specificity of Typing Test

We analyzed a large series of field isolates identified as TSE infected, by a rapid test, in the framework of the French active surveillance network. Of these 270 samples (153 in 2002 and 117 in 2003), 42 samples, which were initially identified by using the TeSeE test, were categorized by AFSSA as atypical due to lack of confirmation by other rapid tests or World Organization for Animal Health–modified SAF immunoblot (AFSSA, no. 2004-SA-0045) (*34*).

When tested with the automated typing test, 10 of the 270 samples had PrPres levels below the detection limit, even undiluted (optical density <0.5 in A conditions). Each series of 32 samples included 3 internal controls (see Table l for results obtained for 20 different series of tests). Among the 2 scrapie controls, 1 (99–1316) was classified as classic as it has a PK resistance similar to most of the field isolates; the second one (PG1259 or 99–1487) was classified as intermediate because its PK resistance was intermediate between classic scrapie and experimental ovine BSE. The distribution of the normalized ratio recorded with the field isolates is shown in Figure 3. The absorbance ratio under the 2 conditions of treatment was remarkably reproducible for the classic scrapie control (mean 1.6 ± 0.2), while larger variations were observed for experimental ovine BSE and the intermediate scrapie control (mean 6.4 ± 0.9 and 3.8 ± 0.5, respectively). These results demonstrate that the present test clearly discriminates classic scrapie strain from the 2 other controls. Because of interassay variations, experimental ovine BSE and intermediate scrapie may slightly overlap (Table 1), further justifying the use of normalized ratio (Figure 2, panel C, and Figure 3).

As shown in Figure 3, only 10 samples (3.8%) provided ratios compatible with experimental BSE (normalized ratio 0.7–1.3), and 28 provided ratios superior to experimental BSE in sheep (normalized ratio >1.3–10.5, Figure 3). Twenty-nine samples, as well as 4 of the 10 samples compatible with experimental BSE and 9 samples with

Table 1. Analysis of the reproducibility of the ELISA typing test*

Sample (N = 20)	Mean ratio A/A′ (normalized)	SD (normalized)	CV, %	95% CI (mean ± 2× SD)
Manual protocol				
Classic scrapie 99-1316	1.3 (0.13)	0.2 (0.02)	15.4	0.9–1.7 (0.09–0.18)
Intermediate scrapie PG1259	4.5 (0.46)	0.8 (0.08)	17.8	2.9–6.1 (0.30–0.63)
Experimental ovine BSE 397BS	9.7 (1.00)	2.0 (0.21)	20.6	5.7–13.7 (0.59–1.41)
Automated protocol				
Classic scrapie 99-1316	1.6 (0.25)	0.2 (0.03)	13.7	1.2–1.9 (0.19–0.30)
Intermediate scrapie 99-1487	3.8 (0.59)	0.5 (0.08)	13.4	2.8–4.8 (0.44–0.75)
Experimental ovine BSE 397BS	6.4 (1.00)	0.9 (0.14)	14.8	4.5–8.3 (0.70–1.30)

*Three samples (classic scrapie 99-1316, intermediate scrapie PG1259 or 99-1487, and experimental ovine BSE 397BS) were analyzed in 20 different experiments using the manual or automated protocol (see Experimental Procedures). Normalized ratios are indicated in parentheses. SD, standard deviation; CV, coefficient of variation; CI, confidence interval; BSE, bovine spongiform encephalopathy.

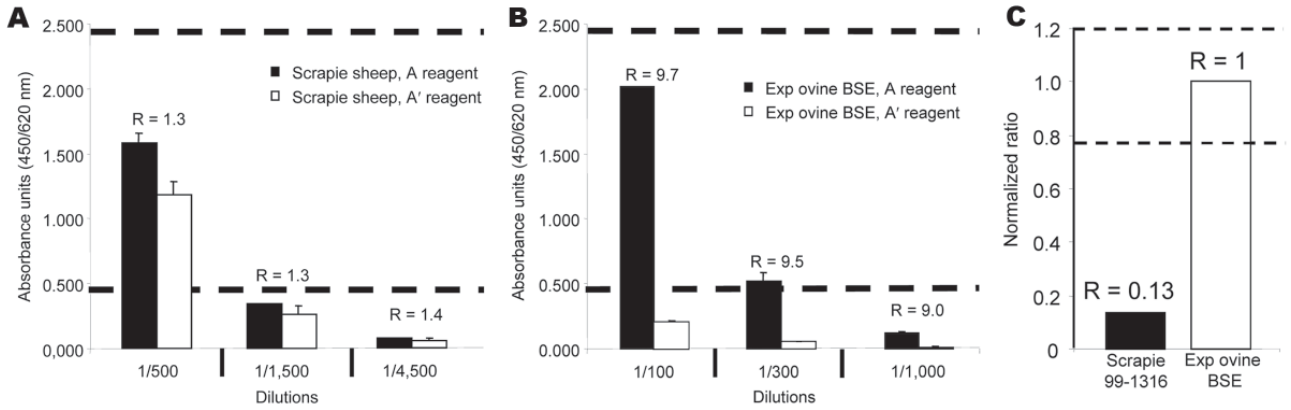

Figure 2. Determination of the A/A' ratio. A dilution series was assayed for each analyzed sample to determine the optimal range that would permit precise determination of the A/A′ ratio (absorbance ranging from 0.5 to 2.5 absorbance units in A reagent). A) A/A′ ratio is close to 1 for PK-resistant prion protein (PrPres) associated with classic scrapie strains (manual protocol, see Experimental Procedures) and B) close to 10 for experimental ovine bovine spongiform encephalopathy (BSE)–associated PrPres. C) To minimize interassay variations, the ratio obtained for each sample is thus normalized by dividing by the ratio obtained for the ovine BSE sample.

low levels of PrPres, were previously identified as atypical scrapie (open bars, Figure 3). Among the samples that yielded ratios compatible with experimental BSE in sheep, 1 goat isolate, Ch636, was extensively studied because of its BSE-like profile in 3 different Western blot techniques (*1*). As shown in Figure 3, this sample had a normalized A/A′ ratio close to 1, very similar to that for experimental goat BSE (Figure 4, panel A, Table 2). This result is confirmed by the migration pattern of the nonglycosylated band (Figure 4, panel B, lane 6), which appears very similar to that of experimental BSE in sheep (Figure 4, panel B, lane 4) and of experimental goat BSE (lane 5), and different from that of French scrapie goat isolates (lanes 7 and 8).

Analysis of Nor98 Isolates

The typing test was used to analyze 18 sheep isolates from Norway (Table 3). Ratios were almost impossible to calculate because of the large decrease in signal in A′ conditions, as shown in Figure 5, panel A for 3 isolates. Only 1 sample (Lavik) showed characteristics of a conventional scrapie isolate, providing an A/A′ ratio of 0.84 (Figure 5, panel A), a normalized ratio of 0.11, and a Western blot profile close to that of a French scrapie isolate (Figure 4, panel B, lanes 3 and 9; Figure 5, panel B, lanes 2 and 4). Other samples had a pattern that included a 12-kDa band (Figure 5, panel B) (*19*,*22*,*34*), characteristic of the Nor-98 strain.

After adapting the conditions of the PK treatment in the second set of measurements (A′ conditions), we observed (see legend, Figure 6) a much lower A/A′ ratio for those Nor-98, which enables discrimination of highly sensitive PK samples (nos. 24 and 26, online Appendix Figure 2 [available from www.cdc.gov/EID/content/14/4/608-appG2.htm] and Table 3) to mildly sensitive PK samples (nos. 8, 11, 16, and 22).

Discussion

When this study was initiated, no case of natural BSE in small ruminants was recorded, and only a few experimental ovine BSE samples were available, all belonging to the same PrP genotype (ARQ/ARQ), and mainly from a first passage. The possible impact of the genotype, the route of infection, and the number of passages on the biochemical properties of PrPres associated with the BSE strain are poorly understood. Now, further data suggest that, at least during the second passage in sheep, the biochemical properties (glycoform pattern in brain) of the BSE agent are unchanged (*35*,*36*). In this study using our ELISA, small ruminant BSE samples clearly behaved differently from conventional scrapie samples. However, slight differences may exist (see ARQ/ARQ vs. ARR/ARR genotype in on-

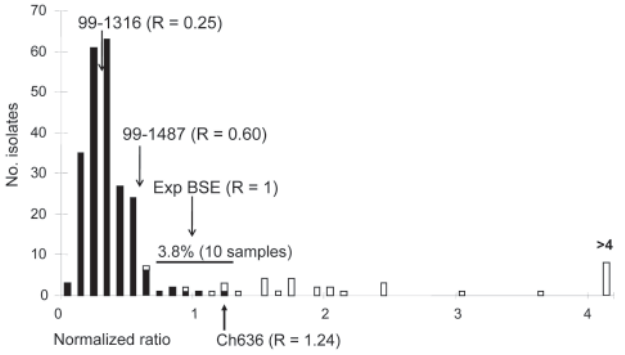

Figure 3. A total of 260 French field-positive isolates were analyzed with the automated ELISA typing test. Each series of 29 samples was analyzed together with 3 internal controls (classic scrapie, intermediate scrapie, experimental ovine bovine spongiform encephalopathy [BSE]). The ratio obtained for each sample was normalized by dividing by the experimental ovine BSE in the same series. Open bars represent the atypical scrapie cases. Natural goat BSE isolate (Ch636) is indicated.

Table 2. Analysis of the experimental caprine BSE samples using the manual ELISA typing test*

Samples	Mean A/A′ ratio (normalized)	SD (normalized)	CV, %	95% CI (mean ± 2×SD)
Experimental BSE goat 1	8.2 (0.75)	1.0 (0.09)	12.2	6.2–10.2 (0.57–0.93)
Experimental BSE goat 2	9.1 (0.83)	1.1 (0.10)	12.1	6.9–11.3 (0.63–1.04)
99-1316	1.5 (0.14)	0.2 (0.02)	13.3	1.1–1.9 (0.10–0.17)
PG1259	4.1 (0.38)	0.6 (0.06)	14.6	2.9–5.3 (0.27–0.49)
Experimental ovine BSE 397BS	10.9 (1.00)	1.8 (0.17)	12.2	7.3–14.5 (0.67–1.33)

*BSE, bovine spongiform encephalopathy; SD, standard deviation; CV, coefficient of variation; CI, confidence interval.

line Appendix Figure 1). We do not know whether these findings reflect differences in the PK sensitivity of PrPres associated with these genotypes or the influence of different tissues.

The main difficulty encountered for the development of a typing test is evaluation of its specificity and sensitivity. In the current study, we unambiguously identified all 37 experimental ovine BSE samples from 25 sheep, including 10 from a second passage. There are few data describing the molecular features of PrPres associated with experimental BSE in goats (*37*,*38*). In the framework of the French scrapie strain-typing network, 18 goats were analyzed by this ELISA, and 2 appeared compatible with experimental ovine and caprine BSE. One of them (Ch636), when analyzed with other molecular typing tests, appeared indistinguishable from experimental BSE and was later confirmed as the first natural case of BSE in a goat (*1*), after experimental transmission in wild-type and transgenic mice. The second BSE compatible sample (TR041528) was later clearly identified as a case of atypical scrapie as defined by its migration pattern (*34*). All these data suggest a good sensitivity for our test, which unambiguously identified all cases of experimental BSE in the sheep and goats tested, as well as the only natural case identified to date in a goat.

Another key point during the development of this test was to ensure good reproducibility because this parameter clearly influences both sensitivity and specificity. Ratios

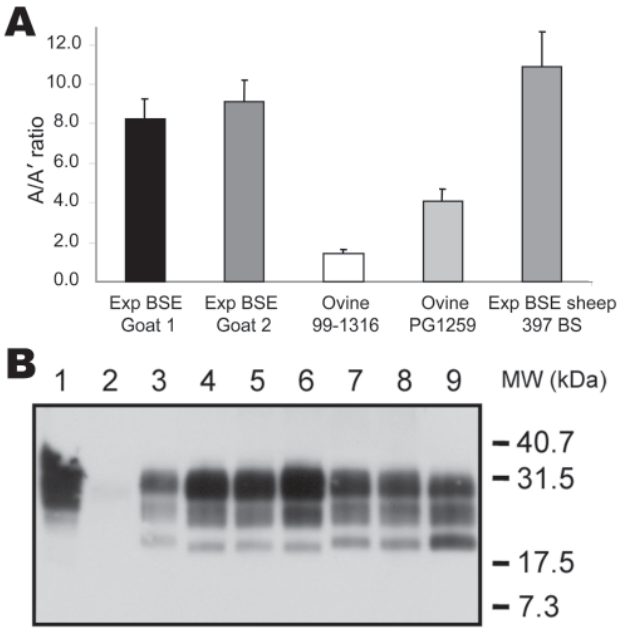

Figure 4. Analysis of goats isolates with ELISA typing test and immunoblot. Two experimental bovine spongiform encephalopathy (BSE) samples in goats analyzed by using the manual typing ELISA (A) gave ratios similar to those of experimental BSE in sheep. Results are the mean of 3 independent experiments. Experimental goat BSE and BSE-like field isolate Ch636 were analyzed by Western blot and compared with scrapie goat isolates (B). Lane 1, untreated negative brain homogenate. Lane 2, proteinase K (PK)–treated negative brain homogenate. Lanes 3–9, PK-treated positive isolates: French scrapie isolate 99–1316 (lane 3); experimental ovine BSE 397 BS (lane 4); experimental BSE goat 2 (lane 5); French goat isolate Ch 636 (campaign 2002) (lane 6); French scrapie goat isolates Ch517 and Ch519 (campaign 2002) (lanes 7 and 8, respectively); Norwegian scrapie isolate (Lavik, lane 9). MW, molecular weight.

Table 3. Normalized ratios obtained for the Norwegian isolates and controls*

No.	Name	Normalized ratio	SD
Isolates			
Nor 98 1	Andoya	2.51	0.58
Nor 98 4	Fiksdal	4.22	1.34
Nor 98 5	Gasbakken	2.73	0.50
Nor 98 6	Hardbakke	3.81	0.76
Scr 7	Lavik	0.42	0.14
Nor 98 8	Lindas	1.43	0.40
Nor 98 9	Lom	5.30	1.37
Nor 98 10	Narvik	10.43	3.56
Nor 98 11	Oppdal	1.79	0.24
Nor 98 12	Rauland	4.28	1.00
Nor 98 13	Rennebu	4.23	0.55
Nor 98 14	Seim	3.16	0.63
Nor 98 16	Stranda	1.90	0.26
Nor 98 17	Torsvastad	3.45	0.33
Nor 98 19	Al	5.31	3.27
Nor 98 20	Arnes	3.99	0.85
Nor 98 21	Aseral	3.20	0.67
Nor 98 22	Egersund	1.68	0.52
Nor 98 24	Soknedal2	14.22	6.21
Nor 98 26	Tennevoll	95.43	90.01
Nor 98 27	Sortland	6.27	1.68
Controls			
Ov 99-1316		0.36	0.11
PG-1259		0.45	0.14
BSE 397BS		1.00	0

*SD, standard deviation; BSE, bovine spongiform encephalopathy.

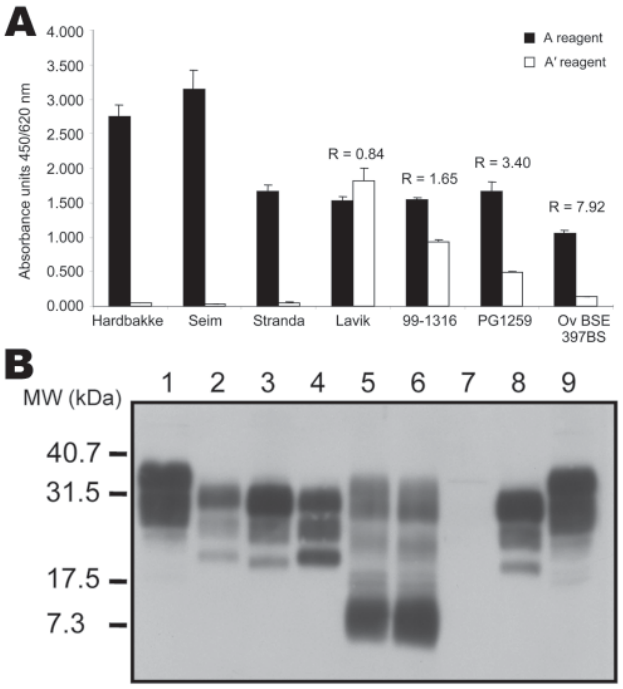

Figure 5. Analysis of different ovine strains by ELISA typing test and immunoblot. A) ELISA typing test. Three Nor98 isolates (Fiksdal, Stranda, and Seim) were analyzed by using the ELISA typing test. Absorbances obtained in the classic A′ typing reagent are close to 0, preventing calculation of the A/A′ ratio. Ratios obtained for a Norwegian scrapie isolate (Lavik) and for the 3 internal controls (classic Scr 99–1316, intermediate scrapie PG1259, and experimental ovine bovine spongiform encephalopathy [BSE] 397 BS) are indicated. B) Pattern of migration of different ovine strains. Lanes 1 and 9, untreated negative brain homogenate. Lanes 2–8, proteinase K–treated brain homogenates: French scrapie isolate 99–1316 (lane 2); experimental ovine BSE 397 BS (lanes 3 and 8); Norwegian scrapie isolate (Lavik) (lane 4); Nor98 Stranda and Nor98 Seim scrapie isolates (lanes 5 and 6, respectively); negative brain homogenate (lane 7). MW, molecular weight.

obtained for the classic scrapie control were highly reproducible, whereas ratios measured for the experimental BSE in sheep and the intermediate scrapie control varied much more, leading to an overlap of the 95% confidence interval (Table 1). To minimize interassay variations, the ratio obtained for each unknown sample was thus normalized by taking as reference the ratio measured for the ovine BSE sample (Figure 2, panel C, and Figure 3) in the same experiment. This enabled us to define the range of normalized ratio compatible with BSE as the mean of experimental ovine BSE ± 2σ on the basis of reproducibility experiments recorded in Table 1. This range was experimentally determined between 0.7 and 1.3, leading to 3 categories for field samples: conventional scrapie (ratio <0.7), compatible with BSE (0.7 <ratio <1.3), and atypical scrapie (ratio >1.3).

Only 10 (3.8%) of the 260 samples analyzed in the framework of the French epidemiologic surveillance network during 2002–2003 gave a ratio compatible with BSE. Of the 10 BSE suspected samples, only 1 goat sample (Ch636) was later confirmed as a true natural BSE case (*1*). This result indicates that the specificity of this test is not that good because 9 false-positive results were recorded in 260 samples (specificity 96.5%). However, the test appears useful since it excluded the presence of BSE for most field samples, thus restricting the use of more specific but time-consuming methods, like experimental transmission in mice, to a small number of isolates. Moreover, in a single screening, this test classified all TSE-infected isolates as a function of their PK resistance and thus provided a rapid

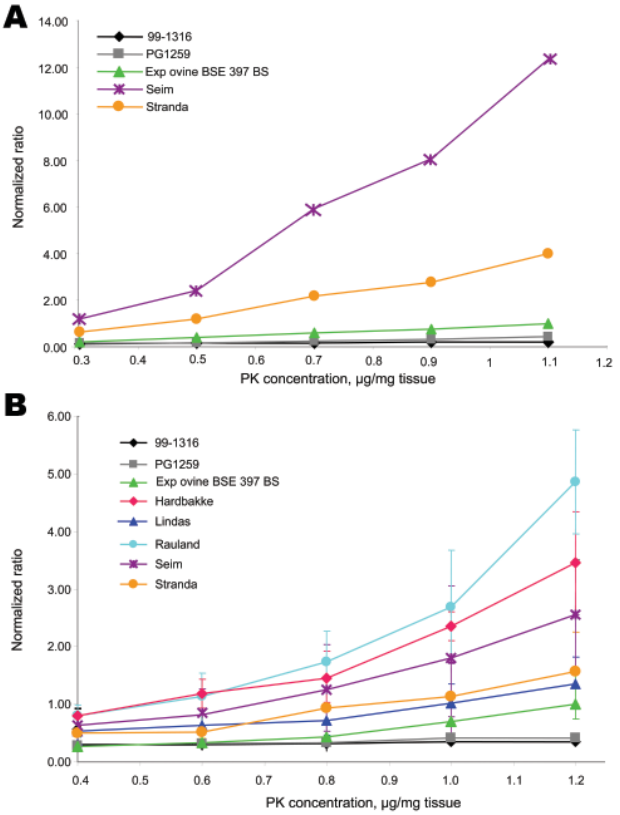

Figure 6. Proteinase K (PK) sensitivity of Nor98 isolates in stringent and mild detergent conditions. The ELISA typing test was performed on Nor98 isolates, with 5 concentrations (0.4–1.1 µg per mg of tissue) in the stringent A′ reagent (A) or in the mild A″ reagent, adapted for PK-sensitive strains (B) (see Experimental Procedures). A/A′ (or A/A″) ratios were calculated for each PK concentration, and normalized by dividing by the A/A' ratio (or A/A″) obtained for the experimental ovine bovine spongiform encephalopathy (BSE) sample at the maximal PK concentration. In the A' reagent, even at the lowest PK concentration (PK 0.4 µg/mg tissue), the normalized ratios (using the experimental ovine BSE A/A′ PK1.1 ratio) obtained for the Nor98 isolates are >1, thus being 3× more sensitive than experimental ovine BSE. To evaluate possible differences in PK sensitivity among Nor98 isolates, this experiment was reproduced with the A″ reagent (panel B), which is 3- to 6-fold more protective than the A′ reagent, as shown by the corresponding normalized ratios (A′ or A″ reagent) for the same PK concentration (1.1 µg PK/mg of tissue).

classification of sheep isolates according to this criterion. The test could also be modified, by adjusting the range of PK sensitivity, to classify Nor-98 isolates.

All these data demonstrate that this ELISA-based typing test is suitable for a routine analysis of field samples, as assessed by the positive evaluation from the European Commission as one of the tests recommended to identify the possible presence of BSE in small ruminant flocks (http://eur-lex.europa.eu/LexUriServ/site/en/oj/2005/l_010/l_01020050113en00090017.pdf). These typing tests are mainly designed to identify the BSE strain in small ruminant flocks. They are performed exclusively in national reference laboratories and based on Western blot techniques. In this context, the present ELISA is one of the secondary tests to be used to confirm BSE suspicion. We believe it will help clarify the status of these unusual isolates.

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Hemorrhagic Fever with Renal Syndrome Caused by 2 Lineages of Dobrava Hantavirus, Russia[1]

Boris Klempa,*†[2] Evgeniy A. Tkachenko,‡[2] Tamara K. Dzagurova,‡ Yulia V. Yunicheva,‡ Vyacheslav G. Morozov,‡ Natalia M. Okulova,‡ Galina P. Slyusareva,‡ Aleksey Smirnov,‡ and Detlev H. Kruger*

Dobrava-Belgrade virus (DOBV) is a European hantavirus that causes hemorrhagic fever with renal syndrome (HFRS); case-fatality rates in Balkan countries are as high as 12%. To determine causative agents, we examined 126 cases of DOBV-associated HFRS in central and southern European Russia. In central Russia (Lipetsk, Voronezh, Orel regions), outbreaks were caused by a DOBV variant (DOBV-Aa) carried by *Apodemus agrarius*. In southern Russia (Sochi district), where HFRS is endemic, HFRS cases were caused by a new DOBV variant (DOBV-Ap), found in *A. ponticus,* a novel hantavirus natural host. Both viruses, DOBV-Aa/Lipetsk and DOBV-Ap/Sochi, were isolated through Vero E6 cells, genetically characterized, and used for serotyping of the HFRS patients' serum. The clinical severity of HFRS caused by DOBV-Aa resembles that of HFRS caused by Puumala virus (mild to moderate); clinical severity of disease caused by DOBV-Ap infections is more often moderate to severe.

In Russia, the zoonotic virus infection with the highest morbidity rate is hemorrhagic fever with renal syndrome (HFRS). This disease was first described in the 1930s as hemorrhagic nephroso-nephritis in far eastern Russia and Tula fever in European Russia (*1*). Since 1978, HFRS has been included in the official reporting system of the Russian Ministry of Public Health. Annually, 10,000–12,000 clinical cases of Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV) infection, mainly characterized by kidney failure, are reported from European Russia. Whereas PUUV infections predominantly occur in urban areas, 97% of DOBV-associated HFRS cases occur in rural environments (E.A. Tkachenko et al., unpub. data).

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The hantaviruses, family *Bunyaviridae*, that cause HFRS are considered emerging viruses because of their increasing importance as human pathogens. Hantaviruses cause 2 human zoonoses: HFRS in Asia and Europe (caused by Hantaan virus [HTNV], Seoul virus [SEOV], PUUV, and DOBV) and hantavirus (cardio)pulmonary syndrome in the Americas (caused by Sin Nombre and Andes viruses) (*2–4*). Recently, 2 novel hantaviruses, with unknown pathogenic potential, were found in Africa (*5*,*6*).

Hantaviruses are transmitted by aerosolized excreta of their natural hosts, mainly rodents (family Muridae) but also shrews (family Soricidae). Particular hantavirus species are usually harbored each by a single or a few closely related rodent species. The virus genome contains 3 segments of negative-stranded RNA; the large (L) segment encodes viral RNA-dependent RNA polymerase, the medium (M) segment encodes glycoprotein precursor, and the small (S) segment encodes nucleocapsid protein (*4*).

DOBV seems to be the most life-threatening hantavirus in Europe; HFRS case-fatality rates are as high as 12% in Slovenia and Greece (*7*,*8*). DOBV was first isolated from a yellow-necked mouse (*Apodemus flavicollis*) captured in a natural focus of HFRS in Dobrava village, Slovenia (*9*). In the 1990s, an outbreak of HFRS in 2 regions of European Russia (Tula and Ryazan) was serologically confirmed to be caused by DOBV, but no clinical characterization of the patients was reported (*10*). In the Tula region, DOBV genetic material was detected in *A. agrarius* (striped field mouse), but not *A. flavicollis,* trapped in Kurkino village, a few kilometers from Tula city (*11*).

[1]This work is dedicated to the memory of our friend and colleague Milan Labuda, who died in August 2007.

[2]These authors contributed equally to this article.

Molecular and serologic evidence indicate that in central Europe DOBV is harbored by *A. agrarius* and causes dozens of HFRS cases per year (*12*–*14*). This particular virus lineage is named DOBV-Aa, and a cell culture isolate of DOBV-Aa has been generated (*14*,*15*). The severity of HFRS caused by DOBV-Aa in central Europe is mild to moderate, less severe than its clinical course in Balkan countries associated with the DOBV-Af variant hosted by *A. flavicollis* (*14*,*16*,*17*).

Another genetic lineage of DOBV was found in the *A. agrarius* species on the Saaremaa island of Estonia, northeastern Europe; the Saaremaa virus has been established in cell culture (*18*). Later, these researchers postulated that Saaremaa virus should represent its own virus species separately from DOBV (*19*). Recently, 3 HFRS patients have been found by serologic approaches to have Saaremaa virus; however, no molecular (nucleotide sequence) identification of the involved virus strains has been reported (*20*).

Detailed phylogenetic analyses show that the strains from *A. flavicollis* form a separate evolutionary lineage (DOBV-Af) and that strains from *A. agrarius* show higher diversity. Strains from central Europe and European central Russia form the DOBV-Aa lineage, which is distinct from Saaremaa strains from northeastern Europe (*12*,*15*). DOBV ecology and evolution have recently become even more complex when DOBV was detected in an additional rodent host, *A. ponticus* (Caucasian wood mouse), captured in Sochi district, in the southern part of European Russia (*21*).

To determine causative agents, we examined 126 HFRS cases from 2 HFRS-endemic areas of European Russia. We isolated the viruses, genetically characterized them, and used them for serotyping.

Material and Methods

Patient Selection

During 2000–2006, blood samples from ≈600 patients around Sochi who had acute febrile illness with suspected hantavirus infection were tested for hantavirus antibody by an indirect immunofluorescence assay (IFA). Of these, 26 patients were found to be hantavirus antibody–positive. During the HFRS outbreak in the Lipetsk region in the winter of 2001–02, hantavirus infection of >100 patients was serologically confirmed by IFA.

Rodent Trapping and Screening

Small mammals were trapped in the Sochi region during the 3 summer and autumn seasons of 2000–2002 and in the Lipetsk region during the winter of 2001–02. Lung tissues of the mammals were screened for the presence of hantavirus antigens by an antigen-capture ELISA as described (*22*).

Virus Isolation

Suspensions (10%) of ELISA-antigen–positive lungs were added to Vero E6 cells as described (*23*). Then, with serum from HFRS patients, the cells were checked for hantavirus antigen by IFA. On the 32nd day of passage, positive cells were detected in a flask containing cells originating from an *A. agrarius* mouse from the Lipetsk region (Aa1854/Lipetsk-02 strain [Aa/Lipetsk]) and on the 70th day in another flask with cells from an *A. ponticus* mouse from Sochi (Ap1584/Sochi-01 [Ap/Sochi]).

IFA

HFRS patient serum was screened for the presence of hantavirus antibody by IFA as described (*24*); slides with combined antigens from Vero E6 cells infected with PUUV, DOBV, HTNV, and SEOV were used as substrates. Slides with monovalent antigens of these viruses were used for serotyping hantavirus antibodies.

Virus Titration and Focus-Reduction Neutralization Test

For confirmation and serotyping, all IFA-positive serum samples were tested by focus-reduction neutralization test (FRNT). The viral stocks, prepared from cell-culture supernatants of infected Vero E6 cells, were titrated with the chemiluminescence focus assay (*25*) or a protein A–peroxidase conjugate/DAB-$NiCl_2$ (*26*). For FRNT, human convalescent serum samples were diluted serially in 2-fold steps, mixed with an equal volume of the respective virus containing 30–100 focus-forming units of this virus, incubated for 1 h at 37°C or overnight at 4°C–6°C, and then used to inoculate the cells. After 6–10 days of incubation, DOBV-N–specific rabbit antiserum or convalescent-phase human serum was used to detect the viral antigen as described above. A reduction in the number of foci of at least 80% was considered as the criterion for virus neutralization.

Reverse Transcription–PCR, Cloning, and Sequencing

Hantavirus RNA was extracted from cell-culture supernatant by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The standard QIAamp viral RNA mini spin protocol was performed. Amplification and sequencing of the entire S and M segments and partial L segment sequences were performed as described for the DOBV SK/Aa isolate (*15*).

Sequence and Phylogenetic Analysis

Sequences were aligned by using ClustalW (*27*). The reliability of the alignment was checked by using DotPlot (*28*). The alignment was tested for phylogenetic information by likelihood-mapping analysis (*29*).

We calculated maximum-likelihood and neighbor-joining phylogenetic trees by using TREE-PUZZLE 5.2

(*30*) and PAUP* 4.0 Beta 10 software packages (*31*), respectively. The Tamura-Nei and Hasegawa-Kishino-Yano evolutionary models with and without gamma distribution of rate heterogeneity were used for the tree reconstructions. We used bootstrap analysis with 10,000 replicates to evaluate the statistical significance of the topology for the neighbor-joining trees. Similarity plots and bootscanning (*32*) were performed by using Stuart Ray's SimPlot 3.2 with default parameters (*33*).

Results

HFRS Cases Associated with DOBV Infections

Altogether we characterized 126 patients with DOBV-associated HFRS (Table 1) from 2 geographically distant areas of European Russia. Of these, 108 were from the Lipetsk region (during 2001–02) and 18 were from the Sochi region (sporadic cases during 2000–2006).

In terms of clinical markers, a significantly higher proportion of the 18 patients from Sochi than from Lipetsk had abdominal pain, vision disturbance, nausea and vomiting, diarrhea, hyperemia of the face, hemorrhagic sclerae, liver enlargement, oliguria, and anuria; 1 patient died. On the other hand, hypertension developed in a significantly higher proportion of the 108 patients from Lipetsk; 1 patient died (Table 2).

The clinical course of the disease was classified as mild, moderate, or severe, following the standard criteria used in the Russian Federation (*34*). According to these criteria, 55% of the cases in the Sochi region were classified as severe, 39% as moderate, and 6% as mild. In contrast, only 27% of the cases in the Lipetsk region were classified as severe, 54% as moderate, and 19% as mild (Table 3).

Association of all investigated HFRS cases with DOBV infection was established by IFA serotyping. Antibody titers against DOBV, HTNV, SEOV, and PUUV were determined. In all cases, antibody titers against PUUV were substantially lower. However, for most HFRS cases, IFA could not differentiate between DOBV, HTNV and SEOV; antibody specificities were finally characterized by FRNT.

Rodent Trapping and Molecular Identification

During 2000–2002, an epizootiologic study was performed to identify and isolate the etiologic agents of the above-described HFRS cases (*21*,*26*). A total of ≈600 small animals (8 species) were trapped during 2000–2002 in the Sochi region and ≈300 animals (10 species) in the winter of 2001–2002 in the Lipetsk region. *A. ponticus* in the Sochi and *A. agrarius* in the Lipetsk region were the species that most frequently carried hantavirus antigen; 19.6% and 57.6% of animals, respectively, were positive according to ELISA.

To ensure correct classification of the reservoir hosts, tissue samples of the 2 animals that served as the sources of virus isolation were subjected to DNA extraction and sequence analysis. Nucleotide sequence of the mitochondrial DNA fragment containing the control region, D-loop, was determined for both animals and compared with *Apodemus* spp. D-loop sequences from GenBank. Neighbor-joining phylogenetic analysis demonstrated that the Aa1854/Lipetsk animal was identified correctly as *A. agrarius*. However, no *A. ponticus* D-loop nucleotide sequence was available in GenBank for comparison. Nevertheless, phylogenetic analysis of Ap1584/Sochi showed that the obtained D-loop sequence was distinct from all other analyzed sequences (Figure 1). This finding at least confirms that Ap1584/Sochi was not a misidentified member of *A. sylvaticus*, *A. flavicollis*, or another morphologically similar *Apodemus* species.

Table 1. Characteristics of 126 patients with Dobrava-Belgrade–associated hemorrhagic fever with renal syndrome, Russia*

Characteristic	Region, %	
	Sochi (2000–2006), n = 18	Lipetsk (2001–02), n = 108
Sex		
M	**94**	**66**
F	**6**	**34**
Age, y		
≤16	12	7
17–59	88	82
≥60	0	11

*__Boldface__ indicates statistically significant differences between groups. Comparison of binomial population proportions analysis implemented in Statlets (NWP Associates, Inc.; www.mrs.umn.edu/~sungurea/statlets/statlets.htm) indicates that the null hypothesis that the 2 proportions are equal could be rejected at significance level of 5.0%.

Table 2. Clinical signs for 126 patients with Dobrava-Belgrade–associated hemorrhagic fever with renal syndrome, Lipetsk (2001–02) and Sochi (2000–2006) regions, Russia*

Selected criteria	Region, %	
	Sochi, n = 18	Lipetsk, n = 108
Average duration of fever, d	7.1	5.4
Abdominal pain	**89**	**46**
Vision disturbance	**12**	**1**
Vomiting	**72**	**27**
Nausea	**89**	**44**
Diarrhea	**50**	**11**
Hyperemia of the face	**72**	**29**
Hemorrhagic sclerae	**50**	**2**
Hypertension	**6**	**34**
Liver enlargement	**83**	**23**
Oliguria (<500 mL)	**77**	**35**
Anuria (<200 mL)	**39**	**8**
Increased blood urea and creatinine	77	81
Death	**5.6**	0.9

*__Boldface__ indicates statistically significant differences between groups. Comparison of binomial population proportions analysis implemented in Statlets (NWP Associates, Inc.; www.mrs.umn.edu/~sungurea/statlets/statlets.htm) indicates that the null hypothesis that the 2 proportions are equal could be rejected at significance level of 5.0%.

Table 3. Severity of clinical disease for 126 patients with Dobrava-Belgrade–associated hemorrhagic fever with renal syndrome, Russia*

Characteristic	Severity†		
	Mild	Moderate	Severe
Clinical sign or symptom			
Maximum temperature, °C	<38.0	38.0–39.5	>39.5
Headache	–/+	+/++	+++/++++
Vision disturbance	–	–/+	+/++
Low-back, abdominal pain	–/+	+/++	+++/++++
Hemorrhagic (petechial) skin rash	–	–/+	–/+/++
Oliguria (minimum mL/d)	>900	300–900	<200–300
Oliguria duration, d	6	9	11–13
Maximum blood urea, mmol/L	<8.3	8.3–19.0	>19.0
Maximum blood creatinine, μmol/L	<130	130–300	>300
Maximum leukocyte count, 10^9/L	<8.0	8.0–14.0	>14.0
Clinical outcome by region			
Sochi (2000–2006)	6%	39%	**55%**
Lipetsk (2001–02)	19%	54%	**27%**

*__Boldface__ indicates statistically significant differences between groups. Comparison of binomial population proportions analysis implemented in Statlets (NWP Associates, Inc.; www.mrs.umn.edu/~sungurea/statlets/statlets.htm) indicates that the null hypothesis that the 2 proportions are equal could be rejected at significance level of 5.0%.
†According to Leshchinskaia et al. (*34*).

Sequence Characterization of Virus Isolates

Complete S- and M-segment and partial L-segment nucleotide sequences of both isolates, Sochi/Ap and Lipetsk/Aa, were determined. The complete S segment of Sochi/Ap was found to be 1,649 nt long. It contained a single open reading frame (ORF; nt 36–1325) that encoded a putative nucleocapsid protein (N) of 429 amino acids. The complete S segment of Lipetsk/Aa was 24 nt longer (1,673 nt) due to a longer 3′ noncoding region. The Sochi/Ap M segment consisted of 3,616 nt that encoded a single ORF (nt 47–3448) of putative 1,133-aa glycoprotein precursor. The first putative start codon at positions 41–43, present in all other DOBV as well as HTNV M segment sequences, was missing, but the next one was located just 6 nt downstream in the same frame (as observed also in SEOV M-segment sequences). The M-segment sequence of Lipetsk/Aa was 3,643 nt long (ORF positions 41–3448; 1,135 aa); the difference in length is again the result of insertions/deletions in the 3′ noncoding region. In addition, a partial L-segment sequence of 541 nt (nt positions 109–649, according to the complete L-segment sequence of DOBV AP/Af; AJ410617) was determined for the Sochi/Ap and Lipetsk/Aa strains.

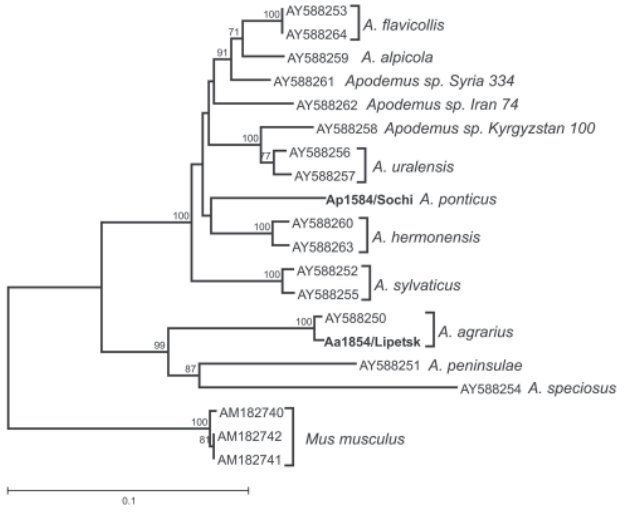

Figure 1. Phylogenetic analysis of D-loop sequences of the animal sources of the viruses Sochi/Ap and Lipetsk/Aa (in **boldface**): *Apodemus ponticus* from the Sochi region (Ap1584/Sochi; EU188455) and *A. agrarius* from Lipetsk region (Aa1854/Lipetsk; EU188456). Sequences of other *Apodemus* spp. were obtained from GenBank; accession numbers are indicated at the branch tips. The neighbor-joining tree was constructed by using the Tamura-Nei (TN93) evolutionary model. Values above the tree branches represent the bootstrap values calculated from 10,000 replicates. The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence.

The sequence similarities between the 2 Russian DOBV isolates were rather low (Table 4). From the existing DOBV cell culture isolates, the Sochi/Ap strain shared the highest similarity with AP/Af19 isolate from Greece. Lipetsk/Aa virus was most similar to the SK/Aa strain. Between other available DOBV sequences, the Sochi/Ap virus S-segment sequence was highly similar to a partial sequence found in an HFRS patient from Krasnodar (P-s1223/Krasnodar-2000) as well as to the sequence Ap-1/Goryachiy Klyuch-2000 amplified from *A. ponticus*. (Krasnodar and Goryachiy Klyuch are places not far from Sochi.) As expected, the Lipetsk/Aa strain was most similar to Kurkino, another *A. agrarius*–associated strain from Russia (Table 4).

Phylogenetic Analysis

Sequences of all 3 segments were analyzed by using maximum-likelihood and neighbor-joining phylogenetic methods with various evolutionary models. If not otherwise stated, all the trees for the particular dataset showed similar

Table 4. Complete nucleotide and amino acid sequence identities of the Sochi/Ap and Lipetsk/Aa strains of Dobrava-Belgrade virus compared with currently available cell culture isolates and most related virus sequences of rodent and human origin*

	Sochi/Ap				Lipetsk/Aa			
	S segment		M segment		S segment		M segment	
Virus isolates	nt	aa	nt	aa	nt	aa	nt	aa
Sochi/Ap	–	–	–	–	86.6	96.7	79.7	91.3
Lipetsk/Aa	86.6	96.7	79.7	91.3	–	–	–	–
SK/Aa	84.8	97.4	78.6	90.4	89.9	98.8	87.2	97.0
Slo/Af	87.8	97.6	79.3	93.3	88.5	96.7	82.7	94.0
AP/Af19	87.6	97.9	79.6	93.3	88.2	97.4	82.5	94.1
Saa/160V	84.4	96.2	78.3	90.2	87.5	96.0	86.3	96.2
Kurkino/53Aa/98	86.6	96.7	NA	NA	98.8	99.5	NA	NA
Ap–1/Goryachiy Klyuch†	96.8	98.8	NA	NA	87.3	96.5	NA	NA
P–s1223/Krasnodar (patient)†	98.7	99.4	NA	NA	86.5	96.4	NA	NA

*NA, not available.
†Values calculated from partial sequences available in GenBank (1,637 bp for Ap–1/Goryachiy Klyuch and 1,196 bp for P–s1223/Krasnodar).

tree topology and statistical support, but only maximum-likelihood trees with Tamura-Nei evolutionary model are shown (Figure 2).

In the S-segment analysis, the Sochi/Ap sequence clustered with high statistical support with the patient-associated sequence from Krasnodar, Russia (AF442623), and the *A. ponticus*–associated sequence from the same region (AF442622) and formed a distinct lineage, which we named DOBV-Ap (Figure 2, panel A). Whereas DOBV-Ap and DOBV-Af share a common ancestor in the S-segment phylogenetic tree, in M and L segment analysis Sochi/Ap formed an outgroup from all other DOBV sequences and did not directly cluster with DOBV-Af sequences (Figure 2, panels B, C). Besides putative genetic reassortment processes, incomplete and unequal sequence datasets (fewer sequences for M- and L-segment datasets are available) could be the reason for these conflicting results. More sequence data are necessary to confirm these findings.

Lipetsk/Aa sequences unambiguously clustered within the DOBV-Aa lineage in the analyses of all 3 segments (Figure 2, panels A–C). As expected, it formed a well-supported monophyletic group with DOBV-Aa strains from Kurkino, Russia. In M and L segment analysis, for which the number of available sequences is rather limited,

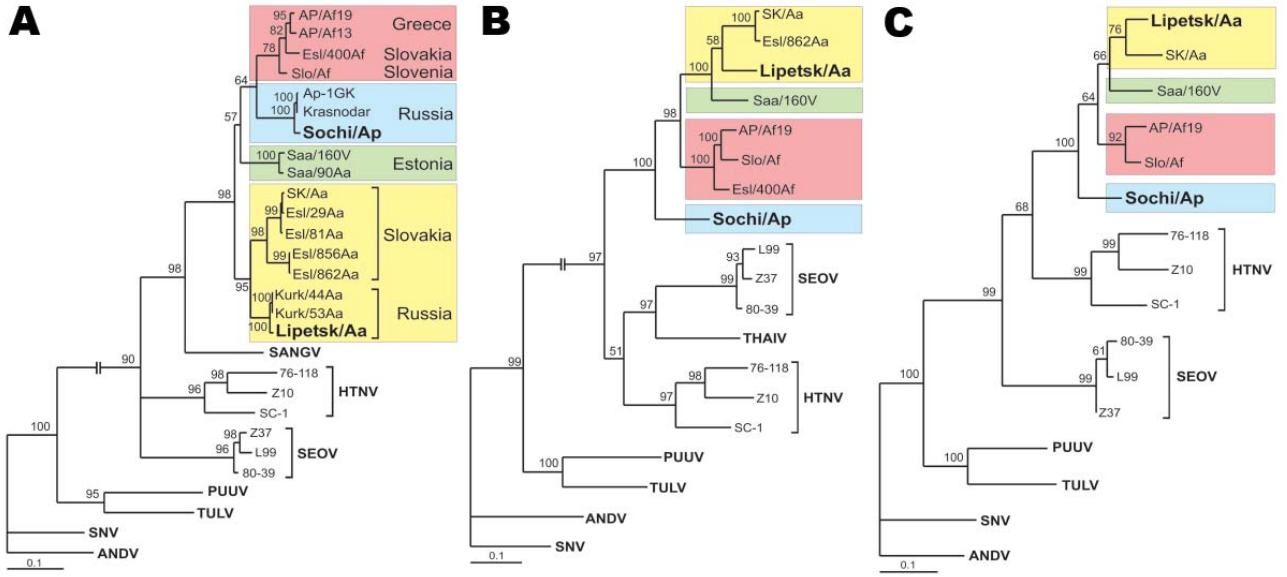

Figure 2. Maximum-likelihood phylogenetic trees of Dobrava-Belgrade virus (DOBV), showing the phylogenetic placement of novel Russian isolates Sochi/Ap and Lipetsk/Aa (in **boldface**) based on A) complete S-segment open reading frame (ORF) (nucleotide sequence positions 36–1325), B) complete M-segment ORF (nt positions 41–3445), and C) partial L-segment sequences (374 nt, positions 157–530). Different DOBV lineages are marked by colored boxes: yellow, DOBV-Aa; green, Saaremaa; red, DOBV-Af; blue, DOBV-Ap (Sochi virus). The Sochi/Ap and Lipetsk/Aa S-, M-, and L-segment sequences were deposited in GenBank under accession nos. EU188449–EU188454. The trees were computed with the TREE-PUZZLE package by using the Tamura Nei evolutionary model. The values at the tree branches are the PUZZLE support values. The scale bar indicates an evolutionary distance of 0.1 substitutions per position in the sequence. SANGV, Sangassou virus; HTNV, Hantaan virus; SEOV, Seoul virus; PUUV, Puumala virus; TULV, Tula virus; SNV, Sin Nombre virus, ANDV, Andes virus; THAIV, Thailand hantavirus.

DOBV-Aa strains from Slovakia were most closely related, although the statistical support for this clustering was below the cutoff value of 70%.

We observed slight differences between maximum-likelihood and neighbor-joining trees in S segment analysis in which Saaremaa strains clustered with DOBV-Af and DOBV-Ap lineages in maximum-likelihood analyses but with DOBV-Aa in neighbor-joining analyses. However, in both instances, the statistical support was below the cutoff limit.

Moreover, we performed recombination analyses by using similarity plots and bootscanning. However, reliable results confirming homologous recombination events that affected Sochi/Ap or Lipetsk/Aa sequences could not be obtained (data not shown).

Serotyping by Using the Novel Virus Isolates

The availability of the 2 novel DOBV strains as cell culture isolates enabled us to use them in chemiluminescence FRNT (c-FRNT) and characterize serum from selected patients also by neutralizing antibody titers. The DOBV prototype strain (Slo/Af; ref.1) and both novel isolates were used in c-FRNT to serotype all 18 patients from the Sochi region (27 serum samples; consecutive samples available for 8 patients). For 15 of these 18 patients, at least 1 serum sample showed the highest neutralizing antibody titer against the local Sochi virus. However, ≥4-fold differences in titers (considered to be significant) were found in serum samples of only 10 patients. For 8 patients, such significant difference was not found (5 patients with 2-fold difference, 3 with no difference). Table 5 shows examples of convalescent-phase serum samples representing these different groups.

From patients in the Lipetsk region, 6 serum samples were characterized by c-FRNT (Table 5). To verify whether some differences can also be found between 2 DOBV-Aa isolates, DOBV strain SK/Aa isolated in Slovakia, Central Europe (*15*), was included in the analysis. Four samples exhibited the highest titer against Lipetsk/Aa when compared with Slo/Af and Sochi/Ap, although in only 2 was the difference 4-fold. One sample reacted equally with Lipetsk/Aa and Slo/Af, and 1 showed even higher reactivity against the Slo/Af strain. When we directly compared Lipetsk/Aa and SK/Aa, equal proportions of serum showed equal titers and 2-fold or 4-fold higher titers against Lipetsk/Aa than against SK/Aa.

In addition, 2 DOBV convalescent-phase serum samples from Slovakia, previously serotyped as anti–DOBV-Af reactive (*15*), were analyzed to determine whether Sochi/Ap, Lipetsk/Aa, and Slo/Af could be distinguished by these samples. In both instances, Slo/Af virus was neutralized best, although in only 1 case was the difference in neutralizing antibody titer 4-fold (Table 5).

Discussion

DOBV circulation was found in the Sochi region, southern part of Russia. We demonstrated that a new DOBV lineage (DOBV-Ap), associated with *A. ponticus* as a novel natural hantavirus host, was a causative agent of the human infection. Second, from an outbreak occurring in the Lipetsk region, central European Russia, >100 HRFS patients were characterized. This outbreak was found to be caused by DOBV-Aa infections. Both viruses, DOBV-Ap/Sochi and DOBV-Aa/Lipetsk, were isolated through Vero E6 cells, genetically characterized, and used for HFRS patient serotyping.

Table 5. Results of typing of neutralizing antibodies in serum from patients with Dobrava-Belgrade virus–associated hemorrhagic fever with renal syndrome, Russia

Region	Sample no.	Time after onset of disease	FRNT titer* against						
			Sochi/Ap	Lipetsk/Aa	Slo/Af	SK/Aa	HTNV	SEOV	PUUV
Sochi (2000–2006)	1,312	104 d	2,560	80	160	ND	160	<80	<40
	3,692	30 d	1,280	160	160	ND	<80	<80	<40
	1,291	16 d	640	160	160	ND	40	40	<40
	4,714	1 y, 5 mo	>20,480	5,120	5,120	ND	320	320	<40
	1,310	50 d	2,560	640	640	ND	160	160	<40
	1,307	15 d	640	320	320	ND	160	160	<40
	4,716	5 y, 3 mo	5,120	1,280	2,560	ND	160	<80	<40
	4,715	1 y, 4 mo	5,120	2,560	5,120	ND	ND	ND	<40
Lipetsk (2001–2002)	4,338	6 mo	160	640	160	320	ND†	ND†	ND†
	3,894	21 d	40	640	40	160	ND†	ND†	ND†
	4,334	3 y, 6 mo	160	640	320	160	ND†	ND†	ND†
	4,344	6 mo	160	640	320	640	ND†	ND†	ND†
	3,958	3 mo	20	80	80	40	ND†	ND†	ND†
	4,329	3 y, 4 mo	640	640	2,560	640	ND†	ND†	ND†
Slovakia	B38	3 y, 9 mo	2,560	640	5,120	2,560	40	640	40
	B39	3 y, 9 mo	2,560	1,280	10,240	2,560	160	160	40

*FRNT, focus-reduction neutralization test. Reciprocal end-point titers are given as determined by chemiluminescence FRNT. ND, not determined.
†Serum previously characterized as anti–Dobrava-Belgrade virus (DOBV) in a first FRNT investigation with Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and DOBV–Slo/Af only.

After the recent detection of DOBV RNA in several *A. ponticus* animals (*21*), isolation of viable virus can be taken as additional evidence that this rodent species represents a novel natural hantavirus host. Sequence and phylogenetic analysis showed that the strains from *A. ponticus* form a distinct lineage, which we propose to call DOBV-Ap. Moreover, clustering of the sequence previously found in a specimen from a patient with severe HFRS in Krasnodar near Sochi (*35*) represents final molecular evidence that DOBV-Ap causes HFRS in this region. The Sochi region is not yet considered to be a DOBV-associated HFRS-epidemic or -endemic region. Our findings therefore have some public health importance because this region is intensively used for recreation. DOBV-associated HFRS should therefore be considered for travelers returning from this region.

The Lipetsk area in Central European Russia is known for the DOBV outbreaks that occurred during 1991–1992 (130 registered cases) and 2001–2002 (167 registered cases, this study). During the winter of 2006–2007, this area faced a new large outbreak, which had ≈600 registered HFRS cases (authors' unpub. data).

Serotyping of neutralizing antibodies confirmed as reasonable the assumptions that the HFRS cases in the Sochi region were caused by DOBV-Ap and that the Lipetsk outbreak was caused by DOBV-Aa strains. In this respect, the differences in clinical courses of infection for the Sochi and Lipetsk patients could be then also assigned as differences in the virulence of DOBV-Ap and DOBV-Aa lineages, respectively. Overall, the clinical course of DOBV-Aa infections in Lipetsk resembles that of PUUV infections observed in Russia (authors' unpub. data), and the DOBV-Ap infections seem more often to be moderate to severe.

However, these differences should not be overestimated. Cases in the Lipetsk region occurred in an outbreak situation in a region where HFRS is a well-known disease. It is therefore possible that physicians and local authorities were much more aware of hantavirus infections and, therefore, also recognized those infections with mild clinical courses. In contrast, the sporadic cases in the Sochi region might be recognized only if the clinical course was severe. Differences in physician awareness in the 2 regions may result in a bias giving the impression that DOBV-Ap infections have a higher clinical severity. Alternatively, the higher virulence of DOBV-Ap might correspond with its close genetic relatedness with the DOBV-Af lineage, which causes rather severe disease in southeastern Europe. At the current stage of knowledge, the order of virulence of DOBV-like viruses in humans seems to be as follows: Saaremaa <DOBV-Aa <DOBV-Ap <DOBV-Af.

Figure 3 shows the regions in Europe where DOBV was demonstrated by serologic as well as molecular analyses to be the causative agent of well-characterized HFRS cases. Region 1 comprises the Balkan area in southeastern Europe where the classic DOBV (our DOBV-Af) was found in *A. flavicollis* animals as well as in human patients (*7*,*8*). Region 2 encompasses northeastern Germany and other regions of central Europe (*13*,*15*) as well as the central part of European Russia (this study) where the DOBV-Aa variant from *A. agrarius* causes mainly mild to moderate HFRS but also severe, life-threatening disease (*17*). The Sochi region, with its novel animal reservoir of DOBV, *A. ponticus*, and the DOBV-Ap–associated HFRS cases is marked as region 3. In all these areas, PUUV also circulates as an HFRS agent.

Figure 3. Map of Europe showing cases, identified by serologic as well as molecular methods, of hemorrhagic fever with renal syndrome caused by infection with the Dobrava-Belgrade virus (DOBV) variants: 1, DOBV-Af; 2, DOBV-Aa ; and 3, DOBV-Ap.

In addition to the public health aspect, our findings add another stone into the very complex mosaic of DOBV ecology and evolution. The Sochi/Ap virus is the first cell culture isolate of novel evolutionary lineage DOBV-Ap. *A. ponticus* is the third rodent species that should be considered a natural host of DOBV. Lipetsk/Aa is a new DOBV strain isolated on cell culture from *A. agrarius* (after Saaremaa virus from Estonia and SK/Aa from Slovakia) and the first originating from Russia and the first isolated in an outbreak region.

Rather unusual for hantaviruses, DOBV has already been found in 3 *Apodemus* species. Nevertheless, other hantaviruses are harbored by >1 (related) host species, e.g., Tula virus has been found in *Microtus arvalis, M. rossiaemeridionalis,* and *M. agrestis* (*36*–*38*) and SEOV in *Rattus rattus* and *R. norvegicus* (*39*). Although the DOBV strains from different *Apodemus* hosts share high amino acid sequence similarity, they can be distinguished in phylogenetic analyses as distinct lineages and seem to possess different virulence in humans as well as in an animal model (*40*).

The novel DOBV-Ap lineage associated with *A. ponticus* emerging in an area south of European Russia confirms the reputation of DOBV being the most virulent of the European hantaviruses.

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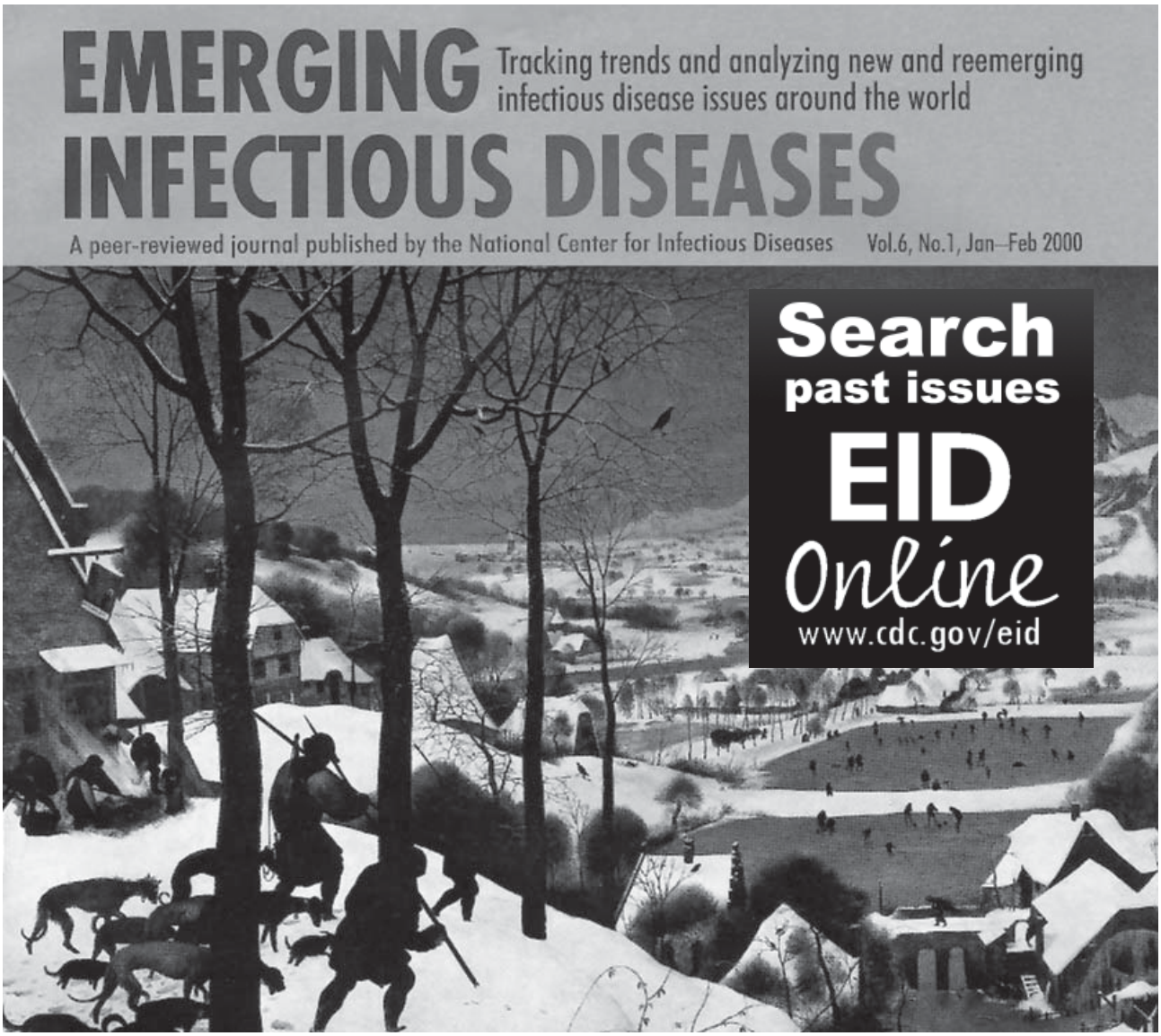

Detection and Prevalence Patterns of Group I Coronaviruses in Bats, Northern Germany

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We tested 315 bats from 7 different bat species in northern Germany for coronaviruses by reverse transcription–PCR. The overall prevalence was 9.8%. There were 4 lineages of group I coronaviruses in association with 4 different species of verspertilionid bats (*Myotis dasycneme, M. daubentonii, Pipistrellus nathusii, P. pygmaeus*). The lineages formed a monophyletic clade of bat coronaviruses found in northern Germany. The clade of bat coronaviruses have a sister relationship with a clade of Chinese type I coronaviruses that were also associated with the *Myotis* genus (*M. ricketti*). Young age and ongoing lactation, but not sex or existing gravidity, correlated significantly with coronavirus detection. The virus is probably maintained on the population level by amplification and transmission in maternity colonies, rather than being maintained in individual bats.

Coronaviruses are enveloped viruses with plus-stranded RNA genomes of 26–32 kb, the largest contiguous RNA genomes in nature (*1*,*2*). They are classified in 3 groups: groups I and II (pathogenic viruses for mammals) and group III (poultry). Group I contains 2 prototypic human pathogenic coronaviruses: human coronavirus (hCoV)-NL63 and hCoV-229E (*3*,*4*). Human pathogenic group II viruses include hCoV-HKU1 and hCoV-OC43 (*5*,*6*). Another human pathogenic coronavirus within a subgroup of group II (termed group IIb) is the severe acute respiratory syndrome (SARS) coronavirus (*7–10*). It caused an international epidemic in 2002 through 2003 that was stopped by a concerted effort that involved strict isolation measures and epidemiologic follow-up (*11*). Although the containment of SARS was a great success in international public health, this and other coronaviruses continue to pose a threat of novel epidemics. The introduction of hCoV-OC43 into humans as a progeny strain of the bovine coronavirus is one example of zoonotic transition of coronaviruses (*12*). Another example is the porcine epidemic diarrhea virus that was introduced into swine in the late 1970s from an unknown source (*13*).

Recently, studies from the People's Republic of China have identified bats as the most likely source of all coronaviruses (*14–18*). Bats constitute ≈20% of all living mammal species (*19*), are distributed on all continents except Antarctica, and occupy diverse ecologic niches in a large range of habitats. Bats exploit a wide dietary diversity, including small vertebrates, nectar, pollen, fruits, blood, and insects (*20*). Almost all bat species live in social groups. The number of bats in such groups ranges from a few up to ≈20 million (*21*), the largest contiguous colonies of mammals on earth (*21*). Typical for all bats is their nocturnal activity and their characteristic roosting behavior during daytime. Roost sites show a great variety that include caves, crevices in rock and tree bark, cavities in tree trunks and branches, foliage, and various human-made structures (*22*). Most bat species regularly use buildings, such as bridges, cellars, mines, wells, and houses, as roosting sites. For this reason, they have contact with humans, possibly enabling virus transmission. Indirect contact by intermediate hosts, such as civet cats and other carnivores, as demonstrated for SARS-CoV, multiply the opportunities of transmission of virus from bats (*23*,*24*).

To predict risks for coronavirus host transition and disease outbreaks, we need to have a deeper understanding of the nature of coronavirus reservoirs, including the

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association of certain coronaviruses with bat species. Unfortunately, bats are extremely difficult to study, and only few studies of sufficient extent have been conducted. Some recent studies indicate that the same coronavirus may be carried by members of the same species of bats in distant locations. At the same time, different species roosting in the same cave may carry different coronaviruses (*18*,*25*).

Outside China, a very recent study described sequences from group I coronavirus in 2 different North American bat species, *Myotis occultus* and *Eptesicus fuscus* (*26*). Together with our recent observation of anti-coronavirus antibodies in African bats (*27*), this finding suggests that the area of distribution of bat coronaviruses may be considerably larger than currently known. In the Western Palaearctic region (Europe, Middle East, North Africa), 50 bats species from 2 suborders and 6 families are known to exist, and the existence of more cryptic species is likely (*28*). We tested bats prospectively for coronaviruses in the context of an ongoing bat-surveillance program in northern Germany; >300 bats were examined. Coronaviruses were detected, sequenced, and analyzed phylogenetically. Strict species association, as well as a likely nonrecent host transition within bats, was detected. From the locations of capture and physical characteristics of bats (age, sex, lactation, gravidity), implications on transmission and maintenance of bat coronaviruses could be drawn in this study.

Figure 1. Map of Germany (inset) with enlarged view of northern Germany. The study area is shaded, and dots in the study area indicate sampling sites.

Materials and Methods

Capture Sites and Sample Collection

Field work was conducted at 8 sites in a 7,834-km^2 area northwest of one of the most important winter roosts of bats in central Europe, the Segeberg Cave (10°18′57″E; 53°56′09″N, Figure 1) in the small town of Bad Segeberg (16,000 inhabitants). The town is located in Schleswig Holstein, the northernmost federal state of Germany. The limestone cave shelters >20,000 bats of up to 8 vespertilionid bat species during hibernation. Bats fly up to 115 km from the hibernation site to their summer roosts in the surrounding landscape (*29*,*30*). Bats were caught with mist nets of different length and height in their foraging habitats where bats hunted insects over water; in forest swarming sites, where bats followed courtship behavior; and near maternity roosts, where only adult females and newborns resided, but no adult males. In our region, different species do not share the same maternity sites. Mist nets were checked at intervals of 5 min. Captured bats were freed from nets immediately and put into cotton bags for several minutes to calm down before further investigations started. Species, age category (juvenile, subadult, adult), sex, reproductive status, forearm length, and body mass were determined. Additionally all pond bats (*M. dasycneme*) were marked with aluminum bands for tracking during an ongoing survey and protection program established by the Schleswig-Holstein Federal Ministry of Environment. In 1 case, a radio transmitter (Holohill, Ontario, Canada) was used for bat tracing. While being kept in bags, bats produced fecal pellets that were collected with clean tweezers and spiked into RNAlater RNA stabilization solution (QIAGEN, Hilden, Germany) for sample processing. Duplicate sampling of bats was prevented by marking the toes of captured bats with nail polish upon first catching. Procedures were consistent with national guidelines for the capture, handling, and care of bats.

Processing and Analysis of Samples

Fecal pellets suspended in RNAlater were homogenized by vortexing. Of the suspensions, 50 μL were introduced into 500 μL of Buffer AVL from the QIAGEN viral RNA minikit and processed further according to the instructions of the manufacturer. Elution volume was 50 μL. Nested reverse transcription–PCR (RT-PCR) was performed exactly as described previously, with primers that provided equally high sensitivity for all coronavirus groups (*31*). Primers targeted a 440-bp fragment of the RNA-dependent RNA polymerase that has been frequently used for phyologenetic comparison of coronaviruses. Because several GenBank entries in this fragment were incomplete, a core region of 334 bp as covered by most GenBank entries was used for analysis. RT-PCR products were sequenced bidirectionally on an ABI 3710 automatic capillary sequencer. Sequences were subjected to nucleic acid alignment by the ClustalW algorithm in the Mega 4 software package (www.megasoftware.net), and analyzed by bootstrapped phylogenetic analysis by using the neighbor-joining algorithm. A nucleic acid distance matrix was also calculated by Mega

4. All analyses on the 334-bp fragment were repeated with the complete 440-bp fragment for validation. All phylogeny and homology results were equivalent, with the exception of a smaller number of complete sequences that were available for the analyses. Results are therefore not shown. All statistical procedures were done with the Statgraphics V 5.1 software package (Manugistics, Dresden, Germany). Sequences from northern German bat coronaviruses can be retrieved from GenBank under accession nos. EU375853–EU375875. Isolation of virus was attempted from diluted RNAlater suspensions, as well as from some fecal pellets suspended separately in phosphate-buffered saline. Vero and CaCo2 cells, as well as primary cell cultures from *Carollia* bat lung and kidney, were used. No virus growth could be confirmed by RT-PCR (data not shown).

Results

From June 1 to August 31, 2007, bats were caught and classified according to species, sex, age category, gravidity, and lactation status. Bat species were typed by morphologic criteria by experienced bat biologists who had worked in the habitat for several years. A total of 315 bats were sampled (Table 1). Overall prevalence of CoV in all bats was 9.8%.

Of the 7 species studied, 5 (*M. dasycneme, M. daubentonii, M. bechsteinii, Pipistrellus nathusii, P. pygmaeus*) yielded coronavirus, with detection rates of 5.2% to 25.4% per species. Detection rates varied significantly between bat species, with *M. dasycneme* showing significantly higher rates than any other species (analysis of variance [ANOVA], $p<0.0002$). Among the *M. dasycneme* bats, detection rates were not equally distributed but correlated significantly with the location in which bats were caught (one-way ANOVA, $p<0.013$). Similar observations were made for 3 other virus-positive species, where detection was achieved in 2 of 6 (*M. daubentonii*), 1 of 3 (*P. nathusii*), and 1 of 4 (*P. pygmaeus*) sampling locations.

Spillover of bats between colonies was not seen, with one interesting exception. In location 2, one of 7 examined *M. dasycneme* bats was already banded. Its ring number showed that it had spilled over from another area. This bat was the only one yielding coronavirus in location 2. By tagging with a radio transmitter, its maternity roost could be traced to location 3, ≈10 km away. When location 3 was sampled, it yielded 45% positive bats (15 of 38 *M. dasycneme* tested), the highest rate of all locations sampled in the study period.

Factors correlating with coronavirus infection were determined. Analysis was conducted on major physical properties. Approximately half of the bats (56%) were females, 30% of them lactating. Twenty-seven percent were juvenile, 12% subadult, and 61% adult. As shown in Table 2, ANOVA analysis identified that young age and ongoing lactation, but not a particular sex or existing gravidity, correlated significantly positively with coronavirus detection. Among female bats, detection rates were significantly higher in those bats associated with maternity colonies than in those caught in foraging habitats (2-tailed *t* test, $p = 0.026$) or swarming sites (2-tailed *t* test, $p = 0.037$). Differences between foraging and swarming sites were not significant ($p = 0.609$).

Sequences of PCR products from all coronaviruses were determined. As shown in Figure 2, the northern German viruses clustered in 1 large monophyletic clade containing no other previously known virus. In a sister relationship was a clade of viruses from Chinese bats with prototype strains A701/2005, HKU6 and A821/2005 (*18*,*34*). These viruses were all detected in *M. ricketti*, which belongs to the same subgenus (*Leuconoe Boie*) as *M. dasycneme* and uses a similar ecologic niche (*35*–*37*).

Within the northern German bat-CoV clade, 4 different lineages appeared to be monophyletically associated with certain bat species. Lineage 1 was associated with *M. dasycneme,* lineage 2 with *P. nathusii,* lineage 3 with *P. pygmaeus,* and lineage 4 with *M. daubentonii.* As expected, the coronavirus from the stray bat from location 2 and the viruses from location 3 clustered closely together in lineage 1. Within the same lineage was 1 coronavirus from an *M. dasycneme* bat that had been sampled at a different site and at a different time (location 5), along with *Pipistrellus* bats that carried clearly distinct virus of lineages 2 and 3. Viruses therefore seemed to be more closely associated with

Table 1. Overview of bats tested for coronaviruses (CoV), Germany*

Species	No. bats (positives)	Females	Juveniles/subadults/adults	Gravid	Lactating	Location†
Myotis bechsteinii	9 (1)	9	4/0/5	0	2	6
M. brandtii	2 (0)	1	0/0/2	1	0	1
M. dasycneme	67 (17)	39	33/1/33	0	22	2,† 3,† 5,† 8
M. daubentonii	155 (8)	79	17/38/100	5	15	1, 2, 4, 5, 7,† 8†
Nyctalus noctula	3 (0)	1	1/0/2	0	1	5
Pipistrellus nathusii	22 (2)	13	15/0/7	0	4	1, 3, 5†
P. pygmaeus	57 (3)	36	15/0/21	6	10	1, 2, 4, 5†

*Name/ type of habitat /geographic coordinates: location 1, Westensee/ f /9°58′17″E/54°16′44″N; 2, Achterwehr/ f /9°57′44″E/54°18′55″N; 3, Methorst/ m /9°49′57″E/54°16′50″N; 4, Molfsee/ f /10°5′22″E/54°17′22″N; 5, Schwentinebrücke/ f /10°17′14″E/54°16′18″N; 6, Bornhöved/ m /10°14′14″E/54°06′04″; 7, Jägerslust/ s /9°55′26″E/54°19′40″N; 8, Projensdorfer Gehölz/ s /10°7′7″E/54°21′59″N.
†Locations in which CoV-positive bats of this species were found.

Table 2. Factors predictive of coronavirus (CoV) detection, Germany

Possible influence factor	Category	% CoV positive	p value*
Age	Juvenile	23.7	0.0015
	Subadult	15.9	
	Adult	8.5	
Sex	Male	17.7	0.39
	Female	14.4	
Lactation	Lactating	22.4	0.021
	Nonlactating	9.7	
Gravidity	Gravid	15.5	0.92
	Not gravid	16.5	

*Indicates level of positive influence on coronavirus detection.

bat species than with sampling locations. On the contrary, virus from the only virus-positive *M. bechsteinii* bat, a very rare and almost extinct species, clustered with lineage 1. Virus detection was successfully repeated from the same sample. These findings suggest that spillover of virus from *M. dasycneme* into *M. bechsteinii* might have occurred. In addition, nonrecent host transition of a common ancestor of *Myotis*-associated CoV into *Pipistrellus* is suggested by virus phylogeny for lineage 2 and 3 viruses (Figure 2).

To appreciate the diversity of northern German bat-CoV, a nucleotide distance matrix of 30 major taxa of coronaviruses was set up, including established coronavirus species and novel bat-CoV taxa, as recently defined (online Appendix Figure, available from www.cdc.gov/EID/content/14/4/625-appG.htm). Lineages 1, 2, and 3 had mutual nucleic acid distances between 6% and 8%. Distance of lineage 4 from the aforementioned was 12%–13%. Distance of northern German lineages 1–4 from the sister clade of Chinese *M. ricketti* CoV was 15%–17%. The Chinese and northern German *Myotis*-associated CoV and their common sister clade, represented by strain A512/2005, were 20%–22% distant. For comparison, among the established species of CoV the lowest degrees of nucleic acid distances were observed between mouse hepatitis virus, human CoV HKU1, and the hCoV-OC43/bovine CoV pair, at 16%–18%. The 2 established lineages within CoV group 2b (SARS-like CoV, bat-SARS CoV HKU3) were 10% distant.

Discussion

Similar to our previous studies on anti-coronavirus antibodies in African bats and recent findings of bat CoV in North America (*26*,*27*), this study shows that the presence of coronaviruses in bats is not a unique phenomenon in Asia and seems to extend worldwide. The prevalence of coronaviruses in bats in northern Germany was 9.8%, which is in the same range as in studies of similar size from China: Lau et al. found 66 (16%) of 412 bats positive for coronaviruses (*38*), Chu et al. found a prevalence of 15.8% (43/272 bats) (*25*), Woo et al. found 4.2% (13/309 bats) (*34*), and Tang et al. found 6.5% (64/985 bats) (*18*).

To explain how coronaviruses might be transmitted and maintained in bat populations, we have statistically determined factors that influence virus detection. Young bats of both sexes, as well as lactating bats, but not gravid bats, were significantly more likely to carry coronaviruses. The virus could be transmitted between young bats and mothers in maternity colonies, rather than circulating year-round at

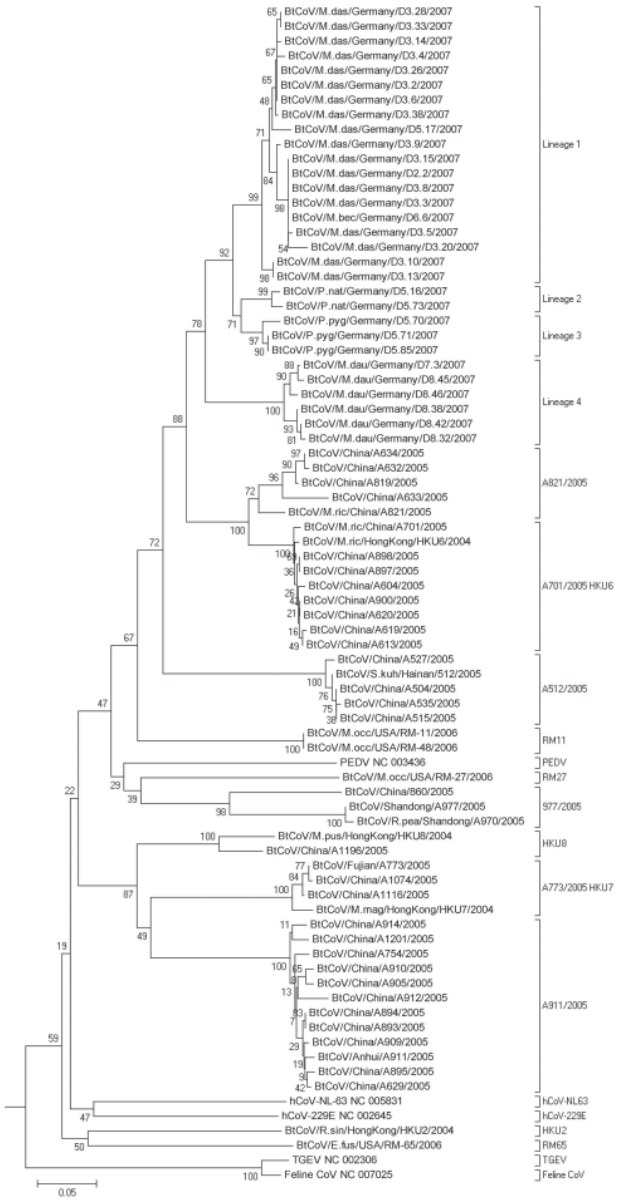

Figure 2. Phlyogenetic analysis of northern German bat coronaviruses (CoV) (lineages 1–4) and related group I CoVs from bats and other mammals. Analyses were conducted in MEGA4 (*32*), by using the neighbor-joining algorithm with Kimura correction and a bootstrap test of phylogeny. Numbers at nodes denote bootstrap values as percentage of 1,000 repetitive analyses. The phylogeny is rooted with a Leopard CoV, ALC/GX/F230/06 (*33*). The column on the right shows bat CoV prototype strain names or the designations of type strains of established mammalian CoV species.

equal levels in the population. Indeed, female bats captured near maternity colonies showed significantly higher virus detection rates than those captured in foraging or swarming sites. Our bats reside in maternity colonies during early summer months; after young bats are born, male bats avoid maternity colonies. One could propose a scenario in which the young provide a susceptible population, amplifying the virus and transmitting it to adult females in maternity colonies. Comparable to many respiratory and enteric virus infections in humans, adults would replicate virus less efficiently than the young because they have at least partial immune protection because of infection earlier in life. This would explain lower detection rates in adult bats. For confirmation, the immune status of young and adult bats against homologous coronaviruses would need to be studied. However, we cannot take blood from our bats without harming them because of their small size; bats in Germany are strictly protected.

Similar to studies conducted in China (*18*,*25*), viruses might be associated with bat species. The same virus was never detected in different species that occurred simultaneously in the same location. For example, *M. daubentonii* and *M. dasycneme* both occurred in locations 2 and 8, but only 1 species per location yielded (different) virus; location 5 harbored all 4 virus-carrying species in 1 place. Three of 4 *Myotis* species yielded viruses (Table 1), and these belonged to 3 different lineages. On the other hand, the same virus lineage was found in remote colonies of the same bat species (compare *M. dasycneme* and *M. daubentonii* in Table 1).

On the local scale of our study, it was difficult to determine whether strict species association or limited local transmission may be responsible for the observed associations is difficult to determine. An influence of local transmission cannot be excluded, considering the hypothesis that virus is likely transmitted in maternity colonies. Groups of bats from the same maternity colony stay together throughout life, and bats of the investigated species never mix in such colonies.

On a larger scale, however, a group I coronavirus hosted by palaearctic (or Old World) *Myotis* bats, including *M. dasycneme* and *daubentonii* in Germany and *M. ricketti* in China (*18*,*34*), might exist. Earlier studies (*18*) used a threshold of 20% nucleic acid distance in our target gene to define a new species of bat coronavirus. By using these criteria, the palearctic *Myotis* virus would form a distinct coronavirus species with German and Chinese subspecies. Even though such a classification is preliminary and does not take other aspects of coronavirus classification into account, it would be supported by host biology. *Myotis* bats do not migrate, but habitats of different *Myotis* species continuously overlap from China throughout Asia into Europe. *M. ricketti*, which harbors the Chinese sister clade of our coronaviruses, belongs to the same subgenus (*Leuconoe Boie*) as *M. dasycneme* and uses a similar ecologic niche (*35*–*37*). A continuous virus population might thus coexist with a continuous palearctic *Myotis* population. As a conclusive extension of this hypothesis, the recently described North American bat coronaviruses RM11 and RM48 from *M. occultus* were more closely related to our viruses than the RM65 strain from an unrelated *Eptesicus fuscus* bat (*26*).

Finally, the virus observed in 2 different *Pipistrellus* species would likely have resulted from a host switch of *Myotis* virus; *Pipistrellus* is not closely related to *Myotis* spp. (*28*). As predicted very recently, coronaviruses may not only be prone to accidental, infrequent host switch between mammals, but may jump from 1 host species to another within the bat reservoir (*39*). Our study supports this notion and suggests that host transition within geographically closely associated, but genetically distinct, bats may have occurred. Because all related viruses in Europe and China were associated with *Myotis*, the direction of transition was probably from *Myotis* spp. into *Pipistrellus* spp., where virus then would have diversified further. This hypothesis is also suggested by relatively long internal branch lengths on the third level of bifurcation (counting from the basal node of the German *Myotis* clade) that separates both *Pipistrellus* virus lineages. This in turn could be a correlate of independent adaptations to *P. nathusii* and *P. pygmaeus*, respectively, after host transition.

Should these initial observations be confirmed in future studies, implications on infection control and prevention of zoonotic outbreaks would be considerable. Targeted eradication of bats is technically impossible and ecologically detrimental. Systematic intervention in the ability of bats to carry coronaviruses might be a realistic, but remote scenario. Further research into the association of coronaviruses with natural hosts is necessary to understand their maintenance patterns and zoonotic potential.

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Multiple Sublineages of Influenza A Virus (H5N1), Vietnam, 2005–2007

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Phylogenetic analysis of influenza subtype H5N1 viruses isolated from Vietnam during 2005–2007 shows that multiple sublineages are present in Vietnam. Clade 2.3.4 viruses have replaced clade 1 viruses in northern Vietnam, and clade 1 viruses have been detected in southern Vietnam. Reassortment between these 2 sublineages has also occurred.

Multiple sublineages of highly pathogenic avian influenza (HPAI) virus (H5N1) have been detected from poultry in Vietnam since 2001 (*1*–*3*). However, the introduction of subtype H5N1 genotype Z virus in 2003 resulted in unprecedented widespread outbreaks in poultry and repeated interspecies transmission to humans; 93 cases were confirmed in Vietnam by the end of November 2005 (*4*,*5*). These viruses became endemic in poultry in Vietnam, causing repeated outbreaks, and have been transmitted to other Southeast Asian countries, where they have caused poultry outbreaks and human infections (*3*–*6*). This virus is designated as clade 1 in the World Health Organization (WHO) influenza (H5N1) virus nomenclature system (*7*). In 2005 a novel reassortant virus of subtype H5N1 (genotype G) from clade 2.3.2 (Mixed/VNM2) was also recognized in Vietnam (*2*). The close phylogenetic relationship of the influenza virus (H5N1) lineages in Vietnam and the southern People's Republic of China suggests repeated introduction of subtype H5N1 virus into Vietnam (*2*,*3*,*8*). However, the development and evolution of influenza virus (H5N1) in Vietnam since 2005 are not clear.

Since 2003, multiple sublineages of the Goose/Guangdong/1/96 (Gs/GD)–like virus became established in poultry in China (*2*). However, in late 2005, clade 2.3.4 (Fujian-like) influenza virus (H5N1) became dominant and replaced almost all of these previously circulating sublineages; these sublineages have also been been detected in wild birds in Hong Kong Special Administrative Region (SAR), China, and from poultry in Lao People's Democratic Republic, Malaysia, and Thailand (*3*). Vaccination of poultry in Vietnam against H5 virus was initiated in October 2005. After that, no influenza (H5N1) outbreaks were reported in the country from December 2005 to October 2006 (*9*). However, renewed subtype H5N1 outbreaks in poultry have occurred in Vietnam since November 2006, with evidence of limited human infections during 2007 and 2008. Whether clade 2.3.4 viruses have been introduced into Vietnam is not known.

The Study

We sequenced the whole genomes of 33 avian influenza virus (H5N1) isolates collected during poultry outbreaks in Vietnam from October 2005 through May 2007. All sequences that were generated in this study have been deposited in GenBank (CY029508–CY029771). The virus was primarily detected in aquatic poultry (ducks, muskovy ducks), but it was also isolated from 2 chickens in December 2006 and January 2007. The date and location of virus isolation are summarized in the Table and Figure 1, panel A.

To understand the developments of influenza virus (H5N1) in Vietnam, we characterized all 8 gene segments of these 33 viral isolates and phylogenetically analyzed them with all available influenza virus (H5N1) previously isolated from Vietnam, Thailand, Malaysia, Lao People's Democratic Republic, and southern China and with reference viruses belonging to each of the designated clades of the WHO influenza (H5N1) nomenclature system. Sequence assembly, editing, multiple sequence alignment, neighbor-joining, and Bayesian phylogenetic analyses were conducted as previously described (*3*). Maximum-likelihood trees were constructed by using Garli version 9.04 (*10*).

The hemagglutinin (HA) genes of all 33 Vietnam isolates were derived from the Gs/GD-like lineage; however, they fell into 3 distinct sublineages (Figure 1, panel B). Seventeen of 33 isolates analyzed were clade 1; however, 15 isolates between March and May 2007 belonged to clade 2.3.4. A single virus isolated in February 2006 (Muscovy duck/Vietnam/1455/2006) clustered within clade 2.3.2.

Phylogenetic analyses also showed a geographic distinction among the isolates characterized in this study. Isolates from samples taken in the northern provinces of Vietnam belonged to clades 2.3.2 and 2.3.4, whereas all isolates in the southern provinces of Vietnam were clade 1 (Figure 1). The clade 1 viruses isolated in the southern provinces from October 2005 through January 2007 were

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Table. Influenza virus isolates from poultry in Vietnam, 2005–2007

Isolate*	Genotype	Date	Province†	Sublineage‡
Dk/VNM/1228/05	Z	2005 Oct	Dong Thap (S)	Clade 1
Dk/VNM/1231/05	Z	2005 Nov	Soc Trang (S)	Clade 1
Dk/VNM/1233/05	Z	2005 Nov	An Giang (S)	Clade 1
MusDk/VNM/1455/06	G	2006 Feb	Ha Tay (N)	Clade 2.3.2
Dk/VNM/1469/05	Z	2005 Nov	Vinh Long (S)	Clade 1
Dk/VNM/1771/05	Z	2005 Oct	Can Tho (S)	Clade 1
Dk/VNM/1/07	Z	2007 Jan	Ca Mau (S)	Clade 1
Dk/VNM/2/07	Z	2007 Jan	Ca Mau (S)	Clade 1
MusDk/VNM/4/07	Z	2007 Jan	Ca Mau (S)	Clade 1
Dk/VNM/5/07	Z	2007 Jan	Ca Mau (S)	Clade 1
Dk/VNM/6/07	Z	2007 Jan	Ca Mau (S)	Clade 1
Dk/VNM/7/07	Z	2007 Jan	Ca Mau (S)	Clade 1
Dk/VNM/8/07	Z	2007 Jan	Bac Lieu (S)	Clade 1
Ck/VNM/15/07	Z	2007 Jan	Soc Trang (S)	Clade 1
Dk/VNM/18/07	Z	2007 Jan	Kien Giang (S)	Clade 1
Ck/VNM/29/07	Z	2006 Dec	Hau Giang (S)	Clade 1
MusDk/VNM/33/07	Z	2007 Jan	Ca Mau (S)	Clade 1
Dk/VNM/34/07	Z	2007 Jan	Can Tho (S)	Clade 1
Dk/VNM/37/07	Z	2007 Mar	Lai Chau (N)	Clade 2.3.4
Dk/VNM/38/07	Z	2007 Mar	Hai Duong (N)	Clade 2.3.4
MusDk/VNM/39/07	Z	2007 Mar	Hanoi (N)	Clade 2.3.4
MusDk/VNM/41/07	Z	2007 Mar	Hanoi (N)	Clade 2.3.4
Dk/VNM/43/07	Z	2007 Apr	Ha Tay (N)	Clade 2.3.4
MusDk/VNM/48/07	Z	2007 May	Hanoi (N)	Clade 2.3.4
MusDk/VNM/49/07	Z	2007 Apr	Hanoi (N)	Clade 2.3.4
Dk/VNM/50/07	Z	2007 May	Hanoi (N)	Clade 2.3.4
MusDk/VNM/51/07	Z	2007 Apr	Ha Nam (N)	Clade 2.3.4
Dk/VNM/52/07	Z	2007 May	Phu Tho (N)	Clade 2.3.4
Dk/VNM/53/07	Z	2007 May	Nghe An (N)	Clade 2.3.4
MusDk/VNM/54/07	Z	2007 May	Hanoi (N)	Clade 2.3.4
Dk/VNM/55/07	Z	2007 May	Hanoi (N)	Clade 2.3.4
MusDk/VNM/56/07	Z	2007 May	Hanoi (N)	Clade 2.3.4
MusDk/VNM/57/07	Z	2007 Apr	Hanoi (N)	Clade 2.3.4

*Ck, chicken; Dk, duck; MusDk, muscovy duck; VNM, Vietnam.
†The letters S and N denote southern Vietnam and northern Vietnam, respectively.
‡Based on the World Health Organization influenza (H5N1) nomenclature system (*7*).

most closely related to viral isolates from poultry in Cambodia in the same period. When one considers the shared land border between Cambodia and southern Vietnam, the close relatedness of these viruses is reasonable. Two of the human influenza (H5N1) cases that were detected from Cambodia were located near the Vietnam-Cambodia border region (*11*). The genetic similarity of strains of influenza virus (H5N1) in Cambodia and southern Vietnam have been observed since 2004 (*4*).

The clade 2.3.2 and clade 2.3.4 viruses isolated from northern Vietnam were most closely related to virus isolated from poultry in Guangxi, China (Figure 1, panel B). Because Guangxi shares a border with Vietnam and trade is extensive between those regions, these viruses were most likely introduced into northern Vietnam through poultry trade. However, the clade 2.3.4 viruses from Vietnam do not form a monophyletic group within clade 2.3.4. Therefore, unlike the single introduction of clade 1 viruses in 2003 (*4*), these results raise the possibility of multiple introductions of the clade 2.3.4 viruses into northern Vietnam.

Phylogenetic analyses of the neuraminidase gene and all internal gene segments (data not shown) show that while most of the isolates were genotype Z viruses, they also formed distinct groups that were broadly similar to the evolutionary relationships seen in the HA tree. Duck/Vietnam/37/2007, which belongs to clade 2.3.4, shared the internal gene constellation of clade 1 viruses (Figure 2), providing evidence for cocirculation of these virus groups and evidence of reassortment between different sublineages within Vietnam influenza (H5N1) isolates. A single influenza (H5N1) genotype G virus (Muscovy duck/Vietnam/1455/2006) was also identified in February 2006, which indicates that genotype G viruses may be persistent in poultry in Vietnam (Figure 2). These results confirm that the genetic diversity of strains of influenza virus (H5N1) in Vietnam is similar to that in southern China during the same period (*3*).

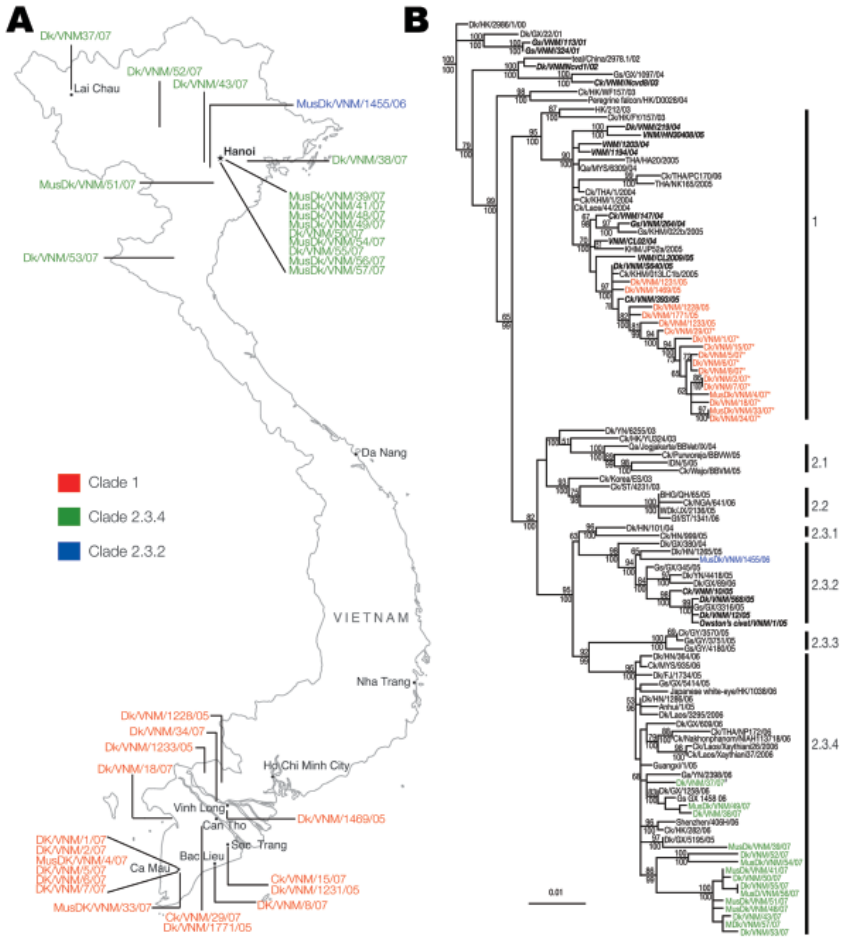

Figure 1. A) Map of Vietnam showing the location of influenza A virus (H5N1) isolation from 2006 to 2007. B) Phylogenetic relationship of the hemagglutinin (HA) gene of influenza A viruses isolated in Vietnam during 2005–2007. Numbers above and below branches indicate neighbor-joining and Bayesian posterior probabilities, respectively. Analyses were based on nt 1–963 of the HA gene. The HA tree was rooted to swan/Hokkaido/51/96. Numbers to the right of the figure refer to World Health Organization influenza (H5N1) clade designations (Table). Virus names described in this study are shown in colored text; previously described Vietnam isolates are shown in ***bold italic*** text. *Denotes viruses with the Ser-123-Pro substitution in the HA. #Denotes a clade 2.3.4 and clade 1 reassortant virus. Scale bar, 0.01 substitutions per site. BHG, bar-headed goose; Ck, chicken; Dk, duck; FJ, Fujian; Gs, goose; GX, Guangxi; GY, Guiyang; HK, Hong Kong Special Administrative Region, People's Republic of China; HN, Hunan; JX, Jiangxi; IDN, Indonesia; KHM, Cambodia; MusDk, muscovy duck; MYS, Malaysia; NGA, Nigeria; Qa, Quail; ST, Shantou; THA, Thailand; VNM, Vietnam; WDk, wild duck; YN, Yunnan.

The motif of multiple basic amino acids at the HA cleavage site that is characteristic of HPAI was maintained in all viruses characterized; however, clade-specific mutations were observed in different sublineages, consistent with previous reports (*3*,*4*,*8*,*12*). The receptor-binding pocket of HA1 retains Gln 222 and Gly 224 (H5 numbering), which preferentially binds avian-like α2,3-NeuAcGal linkages. However, all 12 clade 2.3.4 viruses and the single clade 2.3.2 virus have an Arg-212-Lys mutation in the HA, whereas 12 clade 1 viruses (marked on Figure 1) have a Ser-123-Pro mutation, previously reported from a Vietnam influenza (H5N1) human isolate (*8*), which has been associated with receptor binding. The importance of this change is not clear (*13*,*14*).

Mutations in the Matrix protein 2 ion channel associated with amantadine resistance were detected in all clade 1 virus isolates tested. These viral strains retained the dual mutations Leu26Ile and Ser30Asn in the M2 protein similar to previous clade 1 strains (*15*). No mutation associated with amantadine resistance was recognized in those clade 2.3.2 and clade 2.3.4 strains except the reassortant Dk/VNM/37/07, which had an additional Val-27-Ala mutation in the M2 protein. Thus we recorded an HPAI (H5N1) virus strain with a triple mutation associated with amantadine resistance. All viruses characterized do not have mutations in the NA gene that confer resistance to oseltamivir. Other known virulence mutations, including at polymerase basic protein 2 position 627, were not present in any of the viruses characterized.

Conclusions

This study confirms that clade 2.3.4 virus sublineages that are dominant in southern China have now spread to northern Vietnam (*3*). These viruses appear to have replaced the clade 1 viruses in northern Vietnam just as previous influenza (H5N1) sublineages were replaced in southern China (*3*); however, clade 1 viruses are still detected in the southern provinces of Vietnam. It is, therefore, possible that the clade 1 viruses in southern Vietnam may eventually be replaced by clade 2.3.4. The availability of extensive genetic data from southern China enables us to recognize the development of influenza virus (H5N1) in Vietnam and indicates that clade 2.3.4 viruses may have been introduced into Vietnam on multiple occasions. However, because

systematic surveillance data are lacking, determining the interaction of viruses between the northern and southern provinces of Vietnam, and also between different countries in Southeast Asia, is not possible. When one considers that multiple sublineages of influenza virus (H5N1) are simultaneously endemic to Southeast Asia, systematic surveillance in poultry remains essential to understand the further evolution of this subtype in this region and the potential for pandemic emergence, as well as to monitor the efficacy and cross-protection of poultry vaccines.

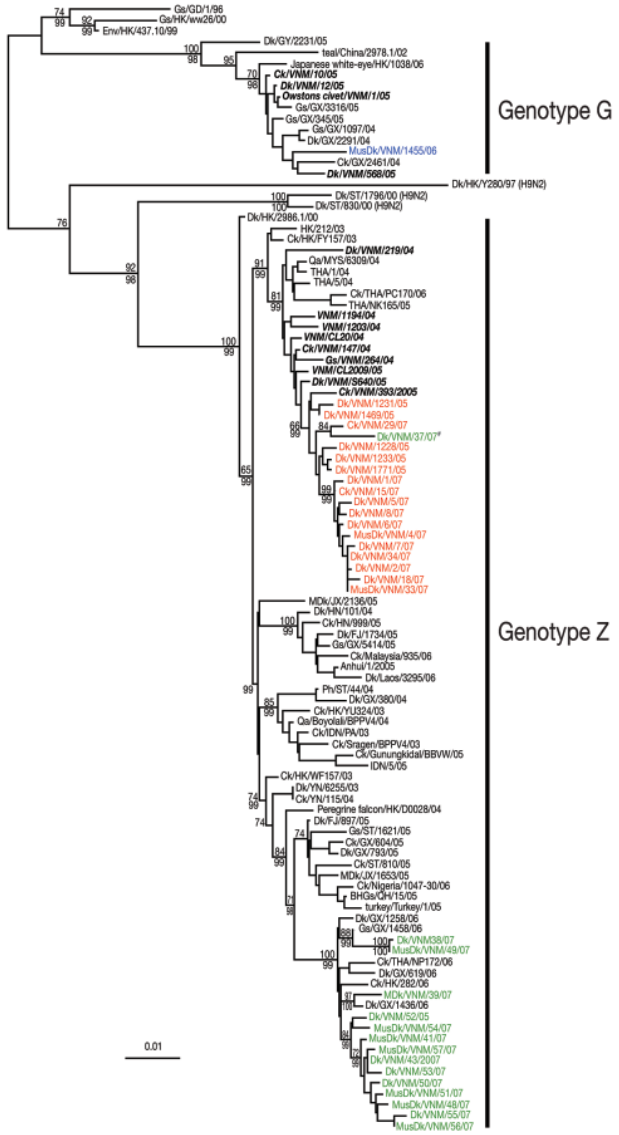

Figure 2. Phylogenetic relationship of the polymerase basic 2 (PB2) gene of influenza A viruses isolated in Vietnam from 2006 to 2007. Numbers above and below branches indicate neighbor-joining and Bayesian posterior probabilities, respectively. Analyses were based on nt 986–2,288 of the PB2 gene. The tree was rooted to equine/Prague/1/56. Viruses names described in this study are shown in colored text; previously described Vietnam isolates are shown in ***bold italic*** text. #Denotes a clade 2.3.4 and clade 1 reassortant virus. Scale bar, 0.01 substitutions per site. BHG, bar-headed goose; Ck, chicken; Dk, duck; FJ, Fujian; Gs, goose; GX, Guangxi; GY, Guiyang; HK, Hong Kong Special Administrative Region, People's Republic of China; HN, Hunan; JX, Jiangxi; IDN, Indonesia; KHM, Cambodia; MusDk, muscovy duck; MYS, Malaysia; NGA, Nigeria; Qa, Quail; ST, Shantou; THA, Thailand; VNM, Vietnam; WDk, wild duck; YN, Yunnan.

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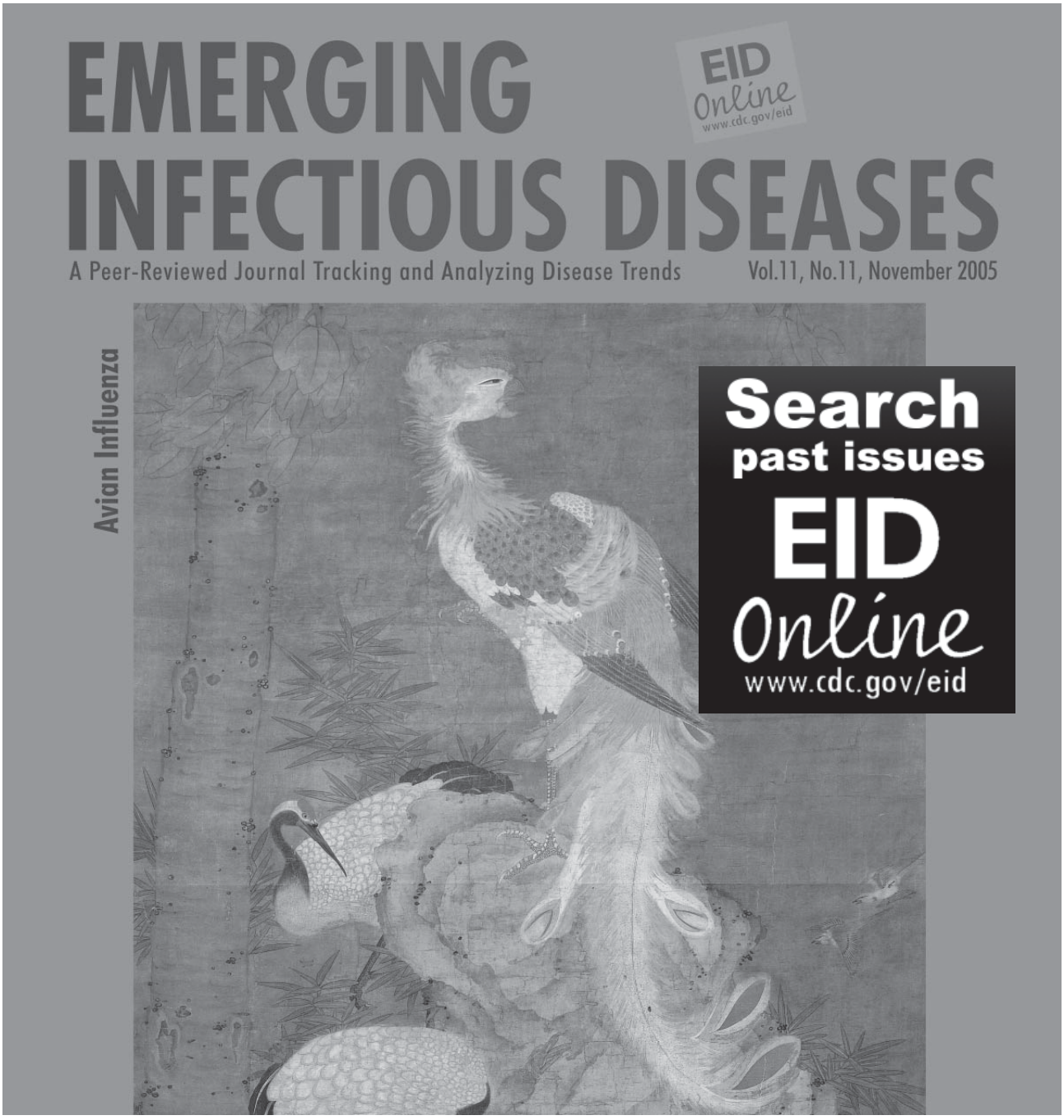

Reassortant Avian Influenza Virus (H5N1) in Poultry, Nigeria, 2007

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Genetic characterization of a selection of influenza virus (H5N1) samples, circulating in 8 Nigerian states over a 39-day period in early 2007, indicates that a new reassortant strain is present in 7 of the 8 states. Our study reports an entirely different influenza virus (H5N1) reassortant becoming predominant and widespread in poultry.

Since its emergence in 2006 in Africa, avian influenza viruses of the H5N1 subtype have spread rapidly to poultry farms in several African countries. In February 2006, Kaduna State in Nigeria was the first of 36 states to report infection of poultry with highly pathogenic avian influenza virus (H5N1). Currently, infection has spread to 22 of the 36 Nigerian states and to the Federal Capital Territory. In February 2007, 1 case of avian influenza was reported in a woman from the southern state of Lagos. Thus, the extensive circulation of influenza virus (H5N1) in Nigeria raises concerns about human and animal health issues. A previous study indicated that 2 sublineages (EMA1 and EMA2) were cocirculating in Nigeria in 2006 (*1*); however, 3 sublineages were identified in a more recent study (*2*), namely sublineage A (corresponding to EMA2) and sublineages B and C (corresponding to EMA1). The 2007 study by Salzberg et al. also identified a virus showing a 4:4 reassortment between genes of sublineages EMA1 and EMA2 (*1*). The aim of our study is to provide additional information on the genetic characteristics of isolates that were circulating in Nigeria in early 2007.

The Study

Twelve representative influenza virus (H5N1) samples from different Nigerian outbreaks were selected (Table 1) by taking into account the geographic origin and the date of isolation. We then characterized these viruses by sequencing the entire genome.

Samples were processed for virus isolation, subtyping, and pathotyping (*3,4*). The amplification of the 8 viral gene segments was carried out with reverse transcription (RT)–PCR by using gene-specific primers (available upon request). PCR products were sequenced in a 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Phylogenetic analysis was performed by using the neighbor-joining method as implemented in the MEGA 3 program (*5*). GenBank accession nos. for the 8-gene segments of the 12 Nigerian strains are EU148356 to EU148451.

As expected, all Nigerian isolates were closely related to the viruses that have been circulating in birds throughout Europe, Russia, Africa, and the Middle East since late 2005. According to the unified nomenclature system for highly pathogenic influenza virus (H5N1), these isolates belong to clade 2.2 (*6*).

Phylogenetic analysis of all 8 gene segments of the recent Nigerian strains showed that 10 of these strains, labeled EMA1/EMA2-2:6 reassortant 2007 (EMA1/EMA2-2:6-R07) (Table 1), have the same genotype. In particular, the genetic comparison of the hemagglutinin (HA) and nonstructural (NS) genes of EMA1/EMA2-2:6-R07 shows that they are derived from viruses of the EMA1 sublineage (*1*) and have the highest similarity with the first Nigerian strain isolated, A/chicken/Nigeria/641/2006 (homology ranged between 99.3% and 99.7%). We observed different topology for the remaining gene segments (neuraminidase, nucleoprotein [NP], matrix, heterotrimeric polymerase complex [PA, PB1, and PB2]). Phylogenetic analysis showed that the nucleotide sequences of these genes fall into EMA2 sublineage; the highest homology was observed with respective gene segments of the Nigerian strains isolated in 2006, which belong to EMA2 (homology ranged between 99.4% and 99.7%). The separation of the gene segments into 2 clusters (Figures 1, 2) is evidence of reassortment (*7*). The genetic pattern of EMA1/EMA2-2:6-R07 virus is distinct from that of A/chicken/Nigeria/1047-62/2006 virus isolated in June, 2006 in Taraba State and recognized previously as an EMA1/EMA2-4:4 reassortant virus (*1*) (Table 2). The remaining 2 viruses were not reassortants. They were detected in Sokoto State and belong to sublineage EMA2.

Sequence analysis showed that the receptor binding site of all of the Nigerian viruses under study retains amino acid residues (Gln 222 and Gly 224). These residues preferentially recognize receptors with saccharides terminating in sialic acid-α2-3-galactose (SAα2-3Gal), specific for avian species (*8*). Mutations that are related to neuraminidase inhibitor and adamantanes resistance were not detected in

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Table 1. List of influenza virus (H5N1) samples analyzed in poultry, Nigeria, 2007

Virus	Group	State of isolation	Date of isolation
A/chicken/Nigeria/1071-1/2007	EMA1/EMA2-2:6-R07	Plateau	Jan 2
A/chicken/Nigeria/1071-3/2007	EMA2	Sokoto	Jan 5
A/chicken/Nigeria/1071-4/2007	EMA1/EMA2-2:6-R07	Borno	Jan 5
A/chicken/Nigeria/1071-5/2007	EMA1/EMA2-2:6–R07	Plateau	Jan 6
A/chicken/Nigeria/1071-7/2007	EMA2	Sokoto	Jan 10
A/chicken/Nigeria/1071-9/2007	EMA1/EMA2-2:6-R07	Bauchi	Jan 12
A/chicken/Nigeria/1071-10/2007	EMA1/EMA2-2:6-R07	Anambra	Jan 13
A/chicken/Nigeria/1071-15/2007	EMA1/EMA2-2:6-R07	Kaduna	Jan 23
A/chicken/Nigeria/1071-22/2007	EMA1/EMA2-2:6-R07	Kano	Jan 31
A/duck/Nigeria/1071-23/2007	EMA1/EMA2-2:6-R07	Borno	Feb 1
A/chicken/Nigeria/1071-29/2007	EMA1/EMA2-2:6-R07	Lagos	Feb 9
A/chicken/Nigeria/1071-30/2007	EMA1/EMA2-2:6-R07	Kaduna	Feb 10

any of the 12 isolates (*9*). All 12 Nigerian viruses possess the PB2 627K mutation associated with increased virulence of influenza virus (H5N1) in mice (*10*). Two other host specific mutations were observed for NP and PA genes. In particular, the NP gene contains a human specific amino acid signature at position 33 (V33I) (*11*) in all selected isolates. A human specific amino acid signature was also detected at position 100 (V100A) of the PA gene of A/chicken/Nigeria/1071-3/2007 and A/chicken/Nigeria/1071-7/2007 strains (*12*).

Conclusions

The results of our study show that 10 of 12 strains obtained over a 39-day period were EMA1/EMA2-2:6-R07 reassortant viruses, and that these were circulating in at least 7 Nigerian states. This appears to be the only report of a reassortant virus generated by H5N1 viruses belonging to the 2.2 clade spreading extensively in poultry. Thus, the viruses circulating in 2007 in Nigeria differ from the original sublineage prototypes introduced during 2006. Our findings also suggest that an influenza virus (H5N1) with

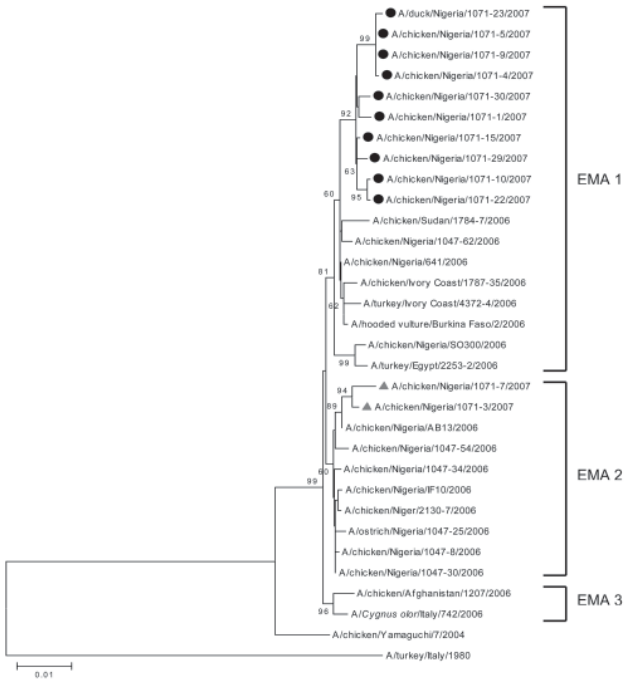

Figure 1. Phylogenetic tree for the hemagglutinin gene of influenza viruses constructed by neighbor-joining method. Sequences obtained in this study were labeled with a circle (EMA1/EMA2-2:6-R07 group) and triangle (EMA2 group). The remaining sequences can be found in GenBank. The numbers at each branch point represent bootstrap values, and they were determined by bootstrap analysis by using 1,000 replications. Scale bar = 0.01 nucleotide substitutions/site.

Figure 2. Phylogenetic tree for nucleoprotein gene of influenza viruses constructed by neighbor-joining method. Sequences obtained in this study were labeled with a circle (EMA1/EMA2-2:6-R07 group) and triangle (EMA2 group). The remaining sequences can be found in GenBank. The numbers at each branch point represent bootstrap values, and they were determined by bootstrap analysis by using 1,000 replications. Scale bar = 0.01 nucleotide substitutions/site.

Table 2. Clustering of the gene segments of influenza strains in poultry, Nigeria, 2007*

Virus	HA	NA	NS	MA	PB2	PB1	PA	NP
A/chicken/Nigeria/641/2006†	EMA1	EMA1	EMA1	EMA1	EMA1	EMA1	EMA1	EMA1
A/chicken/Nigeria/1047-62/2006†	EMA1	EMA2	EMA1	EMA2	EMA2	EMA1	EMA2	EMA1
A/chicken/1071-1/Nigeria/2007 A/chicken/1071-4/Nigeria/2007 A/chicken/1071-5/Nigeria/2007 A/chicken/1071-9/Nigeria/2007 A/chicken/1071-10/Nigeria/2007 A/chicken/1071-15/Nigeria/2007 A/chicken/1071-22/Nigeria/2007 A/duck/1071-23/Nigeria/2007 A/chicken/1071-29/Nigeria/2007 A/chicken/1071-30/Nigeria/2007	EMA1	EMA2	EMA1	EMA2	EMA2	EMA2	EMA2	EMA2
A/chicken/1071-3/Nigeria/2007 A/chicken/1071-7/Nigeria/2007	EMA2	EMA2	EMA2	EMA2	EMA2	EMA2	EMA2	EMA2

*HA, hemagglutinin; NA, neuraminidase; NS, nonstructural; MA; matrix; PB2; polymerase B2; NP, nucleoprotein.
†These strains were analyzed in a previous study (*1*).

new genetic characteristics has emerged in <7 months and is widespread in Nigeria.

The emergence of at least 2 reassortant viruses in Nigeria shows that co-infection with viruses of different sublineages has occurred presumably in poultry. This evidence is most likely a result of poor biosecurity measures implemented by the poultry industry, particularly the live-bird market system, which is known to facilitate mingling of infected birds. In nonindustrialized countries, live bird market systems sometimes allow birds of different species and of unknown health status to share limited space, often the same cage. Birds in the incubation stage or breeds that show a reduced clinical susceptibility may not appear overtly ill and therefore, may be traded in live bird market systems. The movement of infected birds across neighboring regions could explain the genetic relatedness found between influenza virus (H5N1) isolates obtained from 7 Nigerian states. The predominance of a reassortant virus in Nigeria mimicks the previously reported predominance of the Z genotype virus in Asia, although this genotype is believed to contain internal genes originating from non-H5N1 influenza viruses (*13*). The introduction of influenza virus (H5N1) of different clusters in 2005 in Vietnam, also resulted in the emergence of a reassortant strain. Unlike the Nigerian situation described here, the Vietnamese reassortant influenza virus (H5N1) did not become predominant in Vietnam (*1*).

Currently, the genetic characteristics of the human Nigerian isolate are unavailable. Given that human infection occurs after direct contact with infected poultry, studies need to be performed to establish whether the predominant avian influenza virus (EMA1/EMA2-2:6-R07) has also been responsible for human infection. Amino acid mutations towards increased binding to human receptors, namely N182K, Q192R, Q226L, and G228S substitutions in the HA gene (*14,15*), do not seem to have occurred in Nigerian influenza (H5N1) strains to date. However, 2 molecular changes, the 33 NP valine to isoleucine substitution, which is typical of human influenza viruses (*11*), and the 627 PB2 glutamic acid to lysine mutation, which increases virulence in the mammalian host, have been detected in all analyzed strains (*10*).

The results of our study suggest that depopulation and biosecurity measures adopted in Nigeria are not sufficient to prevent the spread of the virus and should be improved. Poultry farmers and smallholder poultry producers (those producing a limited number of poultry) must be educated on appropriate control measures for avian influenza. In addition, isolation and genetic characterization of African influenza virus (H5N1) isolates in a transparent environment, should be promoted and supported so that more information can be gathered on the evolution of this virus in Africa.

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Neuroinvasion by *Mycoplasma pneumoniae* in Acute Disseminated Encephalomyelitis

Bernhard Stamm,* Michael Moschopulos,* Hansjoerg Hungerbuehler,* Jeannette Guarner,† Gillian L. Genrich,† and Sherif R. Zaki†

We report the autopsy findings for a 45-year-old man with polyradiculoneuropathy and fatal acute disseminated encephalomyelitis after having *Mycoplasma pneumoniae* pneumonia. *M. pneumoniae* antigens were demonstrated by immunohistochemical analysis of brain tissue, indicating neuroinvasion as an additional pathogenetic mechanism in central neurologic complications of *M. pneumoniae* infection.

A 45-year-old, previously healthy man had fever, pain in the extremities, nasal discharge, and cough with nonpurulent sputum. He sought clinical care 1 week after onset of illness because of his deteriorating general state, including a headache and paresthesias in both hands. Bilateral basal pneumonia was diagnosed and treated with clarithromycin. During the ensuing 4 days, a rapidly ascending polyradiculoneuropathy resulted in tetraparesis, followed by facial palsy, ophthalmoplegia, and then paralysis of all cranial nerves. The initially fully alert patient became comatose, and assisted respiration was necessary.

On day 9 of the patient's illness, an ELISA (Genzyme Diagnostics Virotech, Rüsselsheim, Germany) was performed on serum samples and showed a *Mycoplasma pneumoniae* immunoglobulin (Ig) G antibody titer of 28.2 Virotech-units/mL (VE) (cut-off 9.0–11.0) and an IgM antibody titer of 20.9 VE (cut-off 9.0–11.0). A PCR for *M. pneumoniae* was positive in tracheobronchial secretions on day 12, and complement fixation test (antigen purchased from Virion CH-8803 Rüschlikon, Zürich, Switzerland) showed *M. pneumoniae* antibody titers of 1,280 (serum) and 4 (cerebrospinal fluid) on day 16.

Serologic tests for cytomegalovirus, Epstein-Barr virus, HIV, measles virus, mumps virus, spring-summer encephalitis virus, *Borrelia burgdorferi*, *Brucella* spp., *Legionella* spp., *Treponema pallidum*, and *Toxoplasma gondii* were negative. No herpes simplex virus 1 or 2 was detected by PCR in cerebrospinal fluid, and PCR results were also negative for *Chlamydia pneumoniae* in tracheobronchial secretions.

On day 8, cerebrospinal fluid examinations showed a total cell count of 43/mm^3 (89% granulocytes and 11% mononuclear cells), total protein 1.3 g/L, and glucose 4.3 mmol/L. On day 15, when the patient was comatose, a total cell count of 794/mm^3 (84% granulocytes and 16% mononuclear cells), total protein 4.6 g/L, and glucose 1.5 mmol/L. Blood values showed leucocytosis with neutrophilia and mild thrombocytosis of 480 g/L.

A computed tomographic scan on day 15 showed brain edema and multiple inflammatory/demyelination lesions in the subcortical white matter of both hemispheres and within the brain thalami, capsulae internae, midbrain, and pons. Electroneurographic and myographic results showed a severe form of a peripheral axonal neuropathy. No antigangliosid (GM) 1 or anti-GM2 antibodies were found in the patient's serum on day 12. We did not look for GQ1b antibodies.

The clinical diagnosis was polyradiculoneuropathy (atypical Guillain-Barré syndrome) and acute encephalitis as complications of bilateral pneumonia caused by *M. pneumoniae*. In addition to clarithromycin, the patient received amoxicillin and ceftriaxone and was given Ig (0.4 g/kg bodyweight/day for 5 days). He died of intractable cerebral edema on day 17 of illness, 10 days after the onset of neurologic symptoms.

At autopsy, the brain was edematous, weighing 1,560 g. The cerebral meninges were macroscopically unconspicuous. On sectioning, multiple hemorrhagic foci with diameters from 0.5 mm to 2 mm were seen within the white matter of the cerebral and cerebellar hemispheres, the brainstem, and also sparsely within the cortex and basal ganglia. There were mild bilateral basal bronchopneumonia and mild hepatic steatosis.

Microscopically, most of the hemorrhagic foci in the brain consisted of fibrinoid necrosis of the wall of small veins, surrounded by hemorrhagic parenchymal necrosis and a dense annular infiltrate of neutrophils and macrophages (Figure). In less disease-advanced areas, zones of acute perivascular periaxial demyelination were seen around intact vessels; within these same areas, a few vessels were associated with a sleevelike infiltrate of T lymphocytes and macrophages with no evidence of demyelination or necrosis, which probably represented an earlier stage of the process.

Multiple levels of the spinal cord had undergone near total necrosis, extending focally into the spinal nerve roots. Perivascular lesions, as observed in the brain, were also present, albeit in smaller numbers. The spinal meninges were focally occupied by a granulocytic infiltrate. Many meningeal vessels were occluded by fresh thrombi. Tissue

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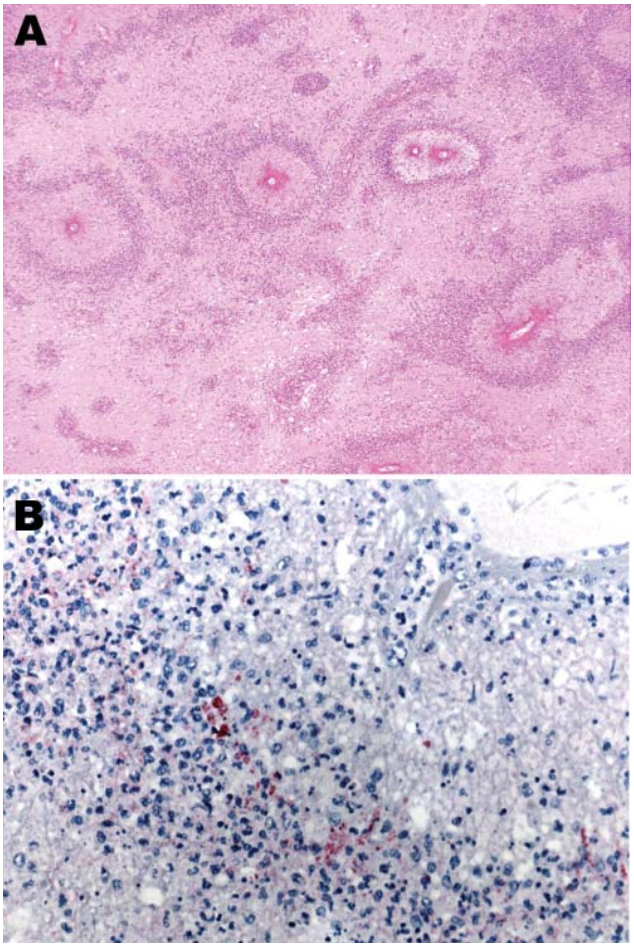

Figure. A) Subcortical cerebral white matter with numerous perivascular foci of demyelination and necrosis (hematoxylin and eosin stain, original magnification ×40). B) Immunohistochemical evidence of *Mycoplasma pneumoniae* antigen inside macrophages present in the perivascular inflammatory infiltrate (immunohistochemical assay performed by using the monoclonal anti–*M. pneumoniae* antibody and naphthol fast red as counterstain, original magnification ×100).

samples of the anterior and posterior spinal nerve roots, spinal ganglia, sciatic nerve, and brachial plexus showed acute widespread demyelination, with a dense infiltrate of CD68-positive macrophages and a mild perivascular infiltrate of T lymphocytes and plasma cells.

Immunohistochemical assays were performed by using a polyclonal antibody (Centers for Disease Control and Prevention, Atlanta, GA, USA), an IgG1 kappa monoclonal anti-*M. pneumoniae* antibody (U.S. Biological, Swampscott, MA, USA; cat. no. M9750–12; dilution 1:25), and an immunoalkaline phosphatase with naphthol fast red chromogenic substrate. *M. pneumoniae* antigens were observed inside macrophages present in the inflammatory infiltrate surrounding necrotic vessels within the cerebral hemispheres (including a biopsy specimen obtained 1 day before death), medulla oblongata, and spinal cord (Figure). No *M. pneumoniae* antigens were found in tissue samples of sciatic nerve and lung, probably because of antigen clearance with time, higher drug levels outside the central nervous system (CNS) as an effect of the blood brain barrier, or both. Anatomic diagnoses were acute disseminated encephalomyelitis; acute, inflammatory, demyelinating polyradiculoneuropathy; necrosis of the spinal cord; brain edema; and disseminated intravascular coagulation.

Neurologic disease is a well-known extrapulmonary complication of *M. pneumoniae* infection (*1*,*2*), manifesting as meningoencephalitis, cerebellar ataxia (*3*), brainstem disease (*4*), transverse myelitis (*5*), polyradiculitis (*4*,*6*), or cranial nerve palsies (*4*,*6*). Our findings and previous anatomopathologic reports (*4*,*6*–*8*) support the generally held view that central neurologic complications are the result of disseminated acute encephalomyelitis (ADEM) or of Hurst syndrome, regarded as a hyperacute variant of ADEM. Congestion and edema of the brain, widespread perivenous demyelination mainly in the cerebral and cerebellar white matter, basal ganglia, brainstem, and spinal cord, and in severe cases, necrosis of vessels and perivascular tissue are the histologic characteristics. Rarely, the demyelinating process extends into spinal nerve roots and peripheral nerves.

The pathogenesis of this syndrome is not fully elucidated. An infection-induced, autoimmune hypersensitivity reaction to myelin proteins is the most frequently advanced hypothesis, suggested by the morphologic similarity to experimental allergic encephalitis and the interval between onset of infection and beginning of neurologic symptoms. A T-cell–mediated, delayed type, hypersensitivity reaction, possibly accompanied by an immune-complex type vasculitis (*4*), seems most probable. The occasional demonstration of circulating antibodies against neural tissue supports this hypothesis. Necrosis of the spinal cord found at autopsy in our patient was probably due to secondary ischemia caused by edema and vascular thrombosis, and a similar process may be responsible for the permanent paraplegia of other reported patients with transverse myelitis after *M. pneumoniae* infection (*5*,*6*).

In recent years, the additional or alternative role of invasion of the CNS by the organism itself has gained renewed interest. *M. pneumoniae* RNA can be detected in brain tissue by nucleic acid hybridization (*8*), and the presence of the organism was demonstrated in cerebrospinal fluid by PCR (*1*,*9*–*12*) and by culture (*3*,*12*). As shown in our case, the microbial antigens can be immunohistochemically detected in histopathologically involved areas of the CNS, both in brain biopsy specimens and at autopsy.

Narita et al. searched for *M. pneumoniae* by PCR in the cerebrospinal fluid of 32 patients; all were <15 years

and had meningitis, encephalitis, or meningoencephalitis after *M. pneumoniae* infection. Positive results were found in 16 of 32 patients. Of the 16 positive results, 15 were seen in samples from 23 patients who had neurologic complications within 7 days after onset of infection, whereas the search was positive in only 1 of the 9 remaining patients with late onset of neurologic complications (*11*,*13*). Bitnun et al. draw a similar conclusion in their review of childhood encephalitis and *M. pneumoniae*, stating that "direct invasion of the CNS is the probable pathogenetic mechanism in children with a brief (<5 days) duration of the prodromal illness" (*9*). In our patient, neurologic symptoms started 7 days after onset of illness.

The organism is likely present, at least in some patients, in the CNS of those who have an early onset of neurologic complications. To assess its pathogenetic role is difficult. The organism may either cause direct damage or trigger a more violent immunologic reaction. Treatment should in that case aim at arriving rapidly at a sufficient concentration of effective antimicrobial agents within the CNS. The fact that neuroinvasion is more prevalent in patients who have an early onset neurologic complications may also relate to necrosis of vessels, a feature of rapidly progressing disease, which may be caused by or facilitate microbial invasion. Prevention, diagnosis, and treatment of neurologic complications in mycoplasmal infections still pose many problems. Immunohistochemistry may contribute to a better understanding of the pathogenesis of the disease and provide insights on clinical management of patients.

Dr Stamm is a general pathologist and head of the Pathology Department at Kantonsspital Aarau since 1990. He is also a professor of pathology at the University of Zürich, Switzerland. His special interests include the pathology of infectious diseases and of the digestive tract.

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Chagas Disease, France

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Chagas disease (CD) is endemic to Latin America; its prevalence is highest in Bolivia. CD is sometimes seen in the United States and Canada among migrants from Latin America, whereas it is rare in Europe. We report 9 cases of imported CD in France from 2004 to 2006.

Nine cases of Chagas disease (CD), although rare in France, have been diagnosed in the country from 2004 through 2006 (online Appendix Table, available from http://www.cdc.gov/EID/content/14/4/644-appT.htm.). These included 1 case of acute Chagas myocarditis (ACM), 4 cases of chronic Chagas cardiomyopathy (CCC), and 4 cases of indeterminate chronic Chagas (ICC) (asymptomatic patients seropositive for *Trypanosoma cruzi*) (*1*). The ACM case involved an otherwise healthy 26-year-old woman who was hospitalized in September 2004 when she returned from a 2-month stay in French Guiana. Her symptoms included fever, headache, photophobia, intermittent chest pain, and arthromyalgia. Physical examination showed a typical Romaña sign, i.e., unilateral periorbital swelling (Figure). No abnormalities were found on clinical workup; blood smears and cultures were negative. Results of lumbar puncture, chest radiography, and echocardiography were also negative. The electrocardiogram (ECG) showed anterior ST-segment depression. A smear of a blister adjacent to the eye showing the Romaña sign yielded *T. cruzi* on direct examination. PCR was not performed. The patient was treated orally with benznidazole, 150 mg twice a day, and had a good clinical response. Benznidazole was discontinued after 7 weeks because peripheral neuropathy had developed. *T. cruzi* serologic results remained negative until 4 months after ACM, either because of a lack of sensitivity or because the patient was treated as soon as possible at the onset of symptoms.

The median age of the other 8 patients (4 men and 4 women from Bolivia) with chronic CD was 38 years (24–48). Seven patients had been living in France for 2 to 5 years. One of the patients with ICC was the son of a woman with CCC. Symptoms were mainly cardiologic, with atypical chest pain, dyspnea (New York Heart Association [NYHA] class 3–4), syncope, lipothymia, and fatigue (online Appendix Table). Two patients were symptom free, including 1 in whom relatively severe cardiac disease was later diagnosed with. Five of these 8 patients had a family history of CCC. Clinically, all patients had bradycardia, hepatojugular reflux, or lower limb edema. Four patients had a normal clinical examination. No anomalies were found (complete blood cell count, transaminases, creatinine phosphokinase, troponin, C-reactive protein). The ECGs of all 4 patients with CCC showed bradycardia, including sinoatrial block (SAB) in 2 patients, and grade III atrioventricular block (AVB) in 2 patients. One patient had a right bundle branch block and a left anterior semiblock. Chest radiographs were normal. Transthoracic echocardiography showed a severe reduction in the left ventricular ejection fraction (20%) in 1 patient. Holter ECG confirmed the conduction abnormalities in 3 of the 4 patients (SAB, AVB, and ventricular hyperexcitability in 2 patients). All 8 patients had a positive indirect immunofluorescence test (IIF) and a positive ELISA test (*2*) for *T. cruzi* in serum (online Appendix Table). The 8 patients were IIF-negative for *Leishmania* (*3*). The 2 patients with AVB III had pacemakers implanted and received angiotensin-converting-enzyme inhibitor and β-blocker therapy. Eight patients received oral benznidazole, 5 mg/kg/day for 1 to 8 weeks, depending on tolerability. Antihistamine therapy was given throughout benznidazole administration. One patient developed DRESS syndrome (drug rash with eosinophilia and systemic symptoms) after 2 weeks of treatment and improved a few days after benznidazole interruption. Nifurtimox was given (and was well tolerated) after the patient's cutaneous and blood status had normalized. Three patients complained of numbness of the extremities during weeks 4, 5, and 7 of treatment; this pointed to benznidazole-induced peripheral neuropathies, which effectively disappeared when treatment was stopped. Three patients stopped taking their treatment prematurely; 1 patient was switched to nifurtimox after 2 weeks of treatment with benznidazole. Two patients reported a lessening of pain and improvements in their general health after antiparasitic treatment.

The prevalence of CD in *T. cruzi*–exposed, asymptomatic persons living in Europe is about 0.6% to 4% (*4,5*). Although CD remains extremely rare in Europe, a review of the literature shows 5 symptomatic cases up to 2004. There was 1 case of imported ACM in France in 1988 in a patient

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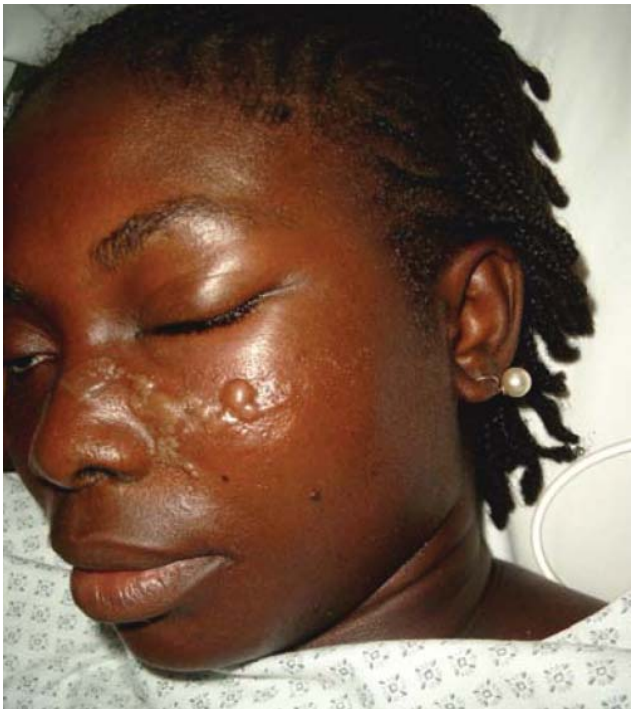

Figure. Romaña sign. Photo of female patient from French Guiana who lives in a metropolitan area of France. She had returned to Maripassoula to visit her parents during the holidays between July 13, 2004, and September 3, 2004. When the patient sought treatment on September 3, 2004, she had fever and unilateral periorbital edema.

from Colombia (*6*), 1 case of autochthonous ACM in Spain in 1992 after blood transfusion (*7*), 1 case of imported CCC in Switzerland in 1996 in a patient from Bolivia (*8*), 1 case of imported ACM in Italy in 1997 in a patient from Brazil (*9*), and 1 case of imported ICC in Denmark in 2000 in a patient from Venezuela (*10*). After 2004, 3 additional cases were reported: 2 cases in Spain in 2005 (1 case of imported CCC in a patient from Bolivia) (*11*), 1 case of autochthonous neonatal ACM in the child of a Bolivian mother (*12*), and 1 case of imported CCC in the Netherlands in 2006 in a patient from South America (country not specified) (*13*).

Acute forms of CD diagnosed in Europe usually involve Europeans returning from stays in disease-endemic areas. The acute case described here underlines, as previously stated by Brisseau et al. (*6*), that a short stay in a disease-endemic zone, even for a few days, is sufficient to become a potential source of *T. cruzi*. In France, since April 2007, all persons who have spent any time in Central or South America are screened for *T. cruzi* before blood donation. This recent measure followed a series of 4 acute Chagas cases in French Guiana (*14*). Chronic imported forms usually involve South American immigrants, whose numbers are difficult to determine in Europe as many are illegal. The number of persons of Latin American origin living in metropolitan France has risen from 27,400 in 1999 to 105,000 in 2005 according to the National Institute for Demographic Studies (www.ined.fr). These persons are an underestimated potential source of transmission of disease. As illustrated by the cases recently reported by C. Riera (*12*), there is also a risk of transplacental transmission in women of South American origin living in Europe. CCC is sometimes life threatening, as in the case of patient 4 (online Appendix Table), who had a very poor cardiac prognosis for a 38-year-old man.

The diagnosis of CD is not always straightforward in France. The current rarity of CD in Europe and the purely cardiologic (and sometimes gastrointestinal) manifestations of the chronic phase represent a diagnostic challenge. In France, few cardiologists and gastroenterologists are fully aware of this infectious disease. In the United States, because imported cases of CD are no longer exceptional, a Chagas screening test for blood donors was implemented in 2007 (*15*). The 9 cases we report, along with other recent cases, may be a sign that CD is emerging in France. If this imported disease becomes established in France, it could represent a real risk for transfusional and congenital transmission, not only in metropolitan areas in France but also in other European countries with a high Latin American immigrant population.

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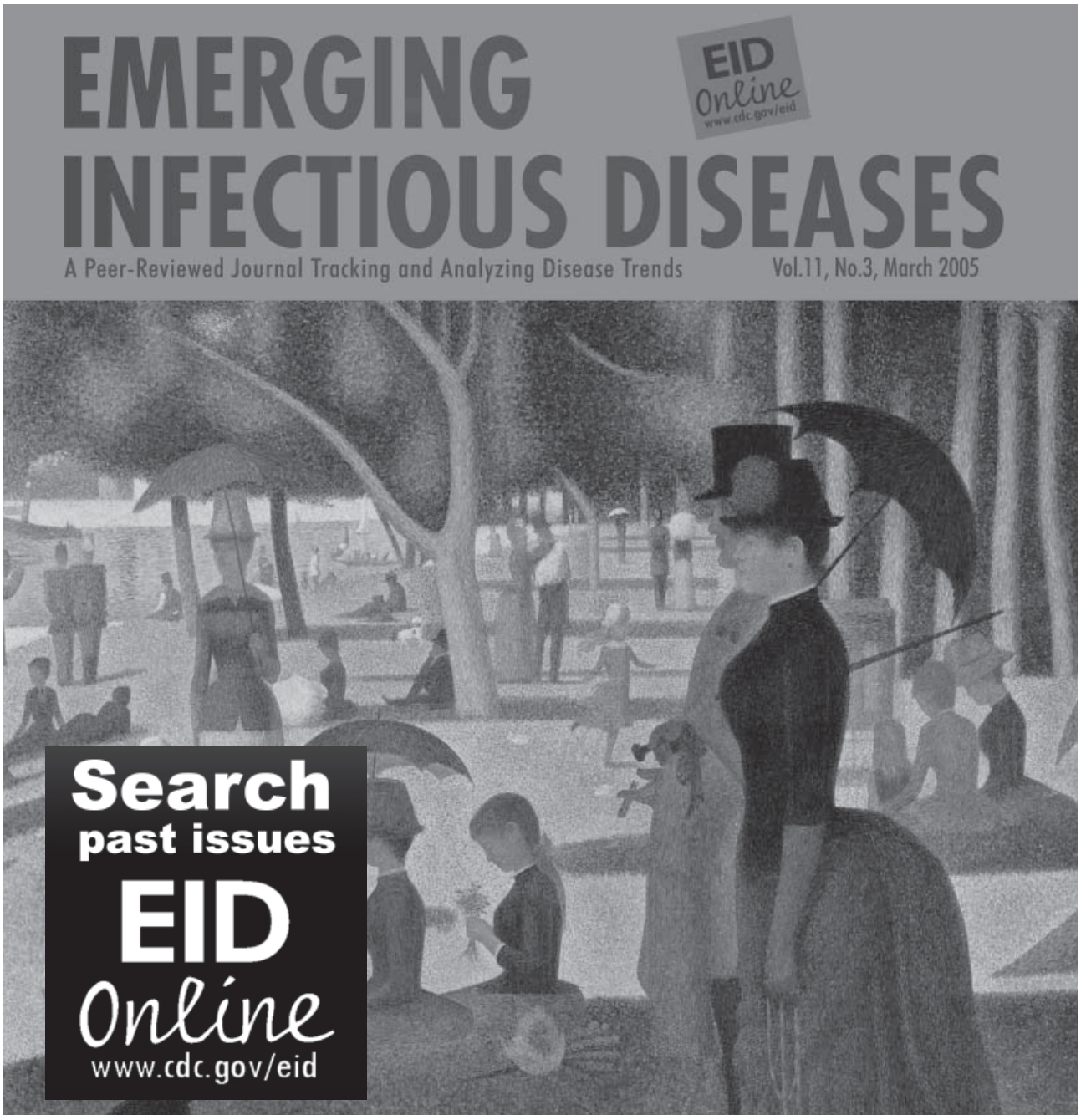

Human Thelaziasis, Europe

Domenico Otranto* and Moreno Dutto†

Thelazia callipaeda eyeworm is a nematode transmitted by drosophilid flies to carnivores in Europe. It has also been reported in the Far East in humans. We report *T. callipaeda* infection in 4 human patients in Italy and France.

Nematodes transmitted by arthropods may cause diseases of different severity, especially in developing countries (*1*). Among these nematodes, *Thelazia callipaeda* Railliet and Henry, 1910 (Spirurida, Thelaziidae) has received little attention. Commonly referred to as eyeworm, it infects orbital cavities and associated tissues of humans, carnivores (i.e., dogs, cats, and foxes), and rabbits (*2*). Because of its distribution in the former Soviet Union and in countries in the Far East, including the People's Republic of China, South Korea, Japan, Indonesia, Thailand, Taiwan, and India (*2*) it has been known as oriental eyeworm. *T. callipaeda* infection is endemic in poor communities in Asia, particularly in China (*3*), where it is frequently reported as being responsible for human thelaziasis with mild to severe symptoms (including lacrimation, epiphora, conjunctivitis, keratitis, and corneal ulcers) (*4*).

A second species, *T. californiensis* Price, 1930, has been reported to infect humans in the United States (*2*). Infective third-stage larvae of eyeworm are transmitted by insects that feed on lacrimal secretions of infected animals and humans that contain *Thelazia* spp. first-stage larvae. In the vector *T. callipaeda*, first-stage larvae undergo 3 molts (≈14–21 days), and infective third-stage larvae may be transmitted to a new receptive host and develop into the adult stage in ocular cavities within ≈35 days (*5*). Competence of drosophilid flies of the genus *Phortica* as vectors of *T. callipaeda* has been recently demonstrated (*6–8*).

Ocular infection of carnivores by *T. callipaeda* has been reported in France (*9*). This infection is also common in dogs (Figure 1) and cats in Italy (*10*). Imported carnivore cases of thelaziasis have also been reported in Germany, the Netherlands, and Switzerland (*11*). The number of case reports of human thelaziasis has increased in several areas of Asia (*3*), where it occurs predominantly in rural communities with poor living and socioeconomic standards and mainly affects the elderly and children. In spite of increasing reports of *T. callipaeda* infection in carnivores in different European countries, no human cases have been described. Thus, infection with this eyeworm is unknown to most physicians and ophthalmologists.

We report autochthonous cases of human thelaziasis in Europe. We sought to raise awareness in the scientific community of the risk for disease caused by this parasite and the need to include this infection in the differential diagnosis of ocular diseases.

The Study

From June 2005 through August 2006, a total of 4 patients with human thelaziasis were referred to the Department of Emergency and Admissions at Croce and Carle Hospital in Cuneo, Italy, for consultation. The 4 male patients (age range 37–65 years) lived in northwestern Italy (43°N, 6°E) and southeastern France (46°N, 9°E), where infections had been reported in dogs, cats, and foxes (*9*,*12*). All patients had similar symptoms (exudative conjunctivitis, lacrimation, and foreign body sensation) for a few days to weeks before referral (Table). All patients required medical attention during the summer (June–August 2005 and 2006) and reported floating filaments on the eye surface. A medical history was obtained for 3 of the patients. The other patient (patient 2, a homeless man) was referred to a physician at the local social services in Nice, France, for severe mental disorders, poor hygiene, and diabetes (Table). Infections in patient 2 were diagnosed 1 month apart in each eye (June and July 2005; referred to as patient 2a and 2b). None of the patients had had any eye disease or had traveled outside their area of residence, with the exception of patient 1 who had gone trekking in the woods in Tenda (Piedmont region, Italy) ≈3 weeks before the onset of symptoms.

Eye examinations showed thin, white nematode(s) on the conjunctival fornix of the affected eye. Nematodes were removed with a forceps after local anesthesia (1% no-

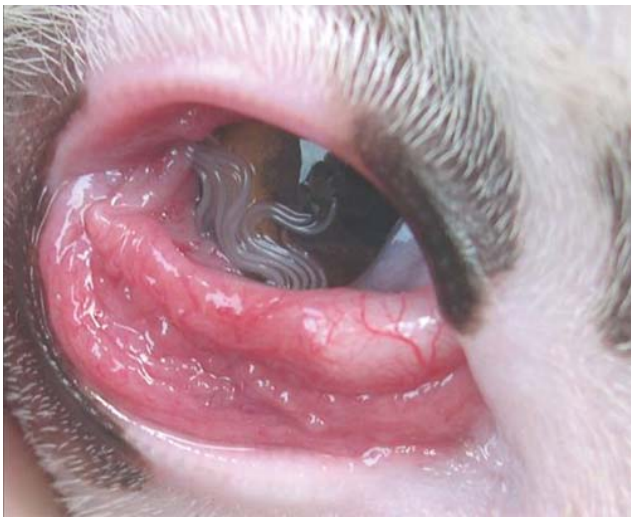

Figure 1. Massive *Thelazia callipaeda* eye infection in a dog.

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Table. Male patients with human thelaziasis, Europe, 2005–2006

Patient no.	Date of symptoms	Location	Age, y	Infected eye	No. and sex of nematodes
1	2005 Jun	Roja Valley, Liguria, Italy	45	Right	1 fourth-stage larva
2a	2005 Jun	Nice, France	65	Right	2 females, 1 male
2b	2005 Jul	Nice, France	65	Left	1 male
3	2006 Aug	Canelli, Piedmont, Italy	48	Right	1 male
4	2006 Aug	Cuneo, Piedmont, Italy	37	Right	1 female

vocaine) was administered. The nematodes were stored in 70% ethanol until they were morphologically identified and analyzed. After the parasites were removed from the eyes, antimicrobial eye drops were prescribed for ≈7 days. Ocular symptoms disappeared within 2–3 days.

Collected nematodes were identified based on morphologic keys (*13*,*14*). *T. callipaeda* nematodes have a serrated cuticle (Figure 2, panel A), buccal capsule, mouth opening with a hexagonal profile, and 6 festoons. Adult females are characterized by the position of the vulva, located anterior to the esophagus-intestinal junction, whereas males have 5 pairs of postcloacal papillae. To confirm morphologic identification, specimens from patients 2 and 4 were analyzed as previously described (*11*). Genomic DNA was isolated from each nematode, and a partial sequence of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1, 689 bp) gene was amplified by PCR. Amplicons were purified by using Ultrafree-DA columns (Amicon; Millipore, Bedford, MA, USA) and sequenced by using an ABI-PRISM 377 system and a Taq DyeDeoxyTerminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were determined in both directions and aligned by using the ClustalX program (*15*). Alignments were verified visually and compared with sequences available for the *cox*1 gene of *T. callipaeda* (GenBank accession nos. AM042549–556).

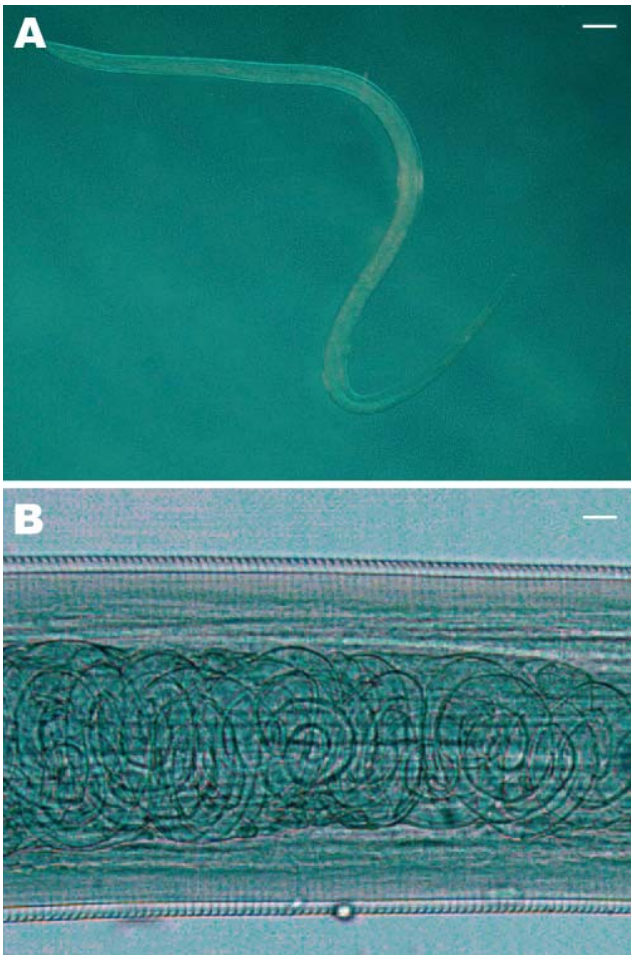

Figure 2. A) Female *Thelazia callipaeda* isolated from patient 4. The posterior end is on the left and the anterior end is on the right (magnification × 200). Scale bar = 500 μm. B) *T. callipaeda* mature first-stage larvae in the distal uterus (magnification ×100). Scale bar = 30 μm.

A total of 6 adult nematodes were morphologically identified as *T. callipaeda* (Table). A mature female nematode (patient 4) had embryonated eggs in the proximal uterus and larvae in the distal uterus (Figure 2, panel B). This suggested that a male worm was present, which had been rubbed out of the eye before symptoms occurred or had remained undetected. Sequences obtained from nematodes were identical to the sequence of haplotype 1 of *T. callipaeda* (GenBank accession no. AM042549) (*11*).

Conclusions

We report human infection by *T. callipaeda* in Italy and France in the same area where canine thelaziasis had been reported. These infections highlight the importance of including this arthropod-borne disease in the differential diagnoses of bacterial or allergic conjunctivitis. All cases of human thelaziasis were reported during the summer months (June–August), which is the period of *T. callipaeda* vector activity (late spring to fall in southern Europe) (*7*). The seasonality of human thelaziasis may impair correct etiologic diagnosis of this disease because spring and summer are the seasons in which allergic conjunctivitis (e.g., by pollens) occurs most frequently. This finding is particularly important when infections are caused by small larval stages that are difficult to detect and identify. Furthermore, clinical diagnosis of human thelaziasis is difficult if only small numbers of nematodes are present because clinical signs related to an inflammatory response mimic allergic conjunctivitis, especially when they are associated with developing third- or fourth-stage larvae.

Untimely or incorrect treatment of the infection may result in a delay in recovery, mainly in children and the elderly, who are most likely to be exposed to infection by the fly. Although treatment for canine infection with *T. callipaeda* with topical organophosphates, 1% moxidectin, or a formulation containing 10% imidacloprid and 2.5% moxidectin is effective, mechanical removal of parasites in humans remains the only curative option (*3*). Thus, prevention of human thelaziasis should include control of the fly vector by use of bed nets to protect children while they are sleeping and by keeping their faces and eyes clean. Genetic identification of haplotype 1 has shown that this is the only haplotype circulating in animals (i.e., dogs, cats, and foxes) in Europe (*11*). This finding confirms the metazoonotic potential of *Thelazia* spp. infection and the need to treat infected domestic animals, which may act as reservoirs for human infection.

Acknowledgment

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Rabies Virus in Raccoons, Ohio, 2004

J. Caroline Henderson,*[1] Roman Biek,*[2] Cathleen A. Hanlon,† Scott O'Dee,‡ and Leslie A. Real*

In 2004, the raccoon rabies virus variant emerged in Ohio beyond an area where oral rabies vaccine had been distributed to prevent westward spread of this variant. Our genetic investigation indicates that this outbreak may have begun several years before 2004 and may have originated within the vaccination zone.

Several wild carnivorous mammals may be competent zoonotic reservoirs for rabies viruses (*1*). Similar to how parenteral vaccination has contributed to control and elimination of rabies in dogs, effective oral rabies vaccines and application methods for wildlife species, most notably the red fox (*Vulpes vulpes*), have led to regional containment and elimination of the rabies virus variants associated with this species in large parts of Canada and Europe (*2*). The first step toward reducing the size of areas in which rabies is enzootically transmitted is containment of its regional spread. Understanding the conditions under which containment of wildlife rabies can reliably be achieved will facilitate the long-term goal of eliminating particular rabies virus variants from their respective reservoir species.

During the late 1970s, the range of a raccoon (*Procyon lotor*)–specific rabies virus variant (RRV) expanded substantially from the historically affected southeastern United States to the currently affected eastern North America (*3*). In 1996, to contain westward expansion of this variant, oral rabies vaccine (ORV) was distributed in Ohio. The ORV strategy includes distributing bait containing a vaccinia-rabies glycoprotein recombinant vaccine (*4*) while taking advantage of physiogeographic impediments to rabies transmission, such as mountains, rivers, and major highways to create a barrier 50 km–150 km wide between unaffected and enzootic areas.

During 1999–2004, ORV had apparently limited further spread of the virus (*5*) (Figure 1). However, in July 2004, RRV was diagnosed in a raccoon northwest of the ORV zone in Lake County, Ohio. As of December 2005, enhanced surveillance had detected 77 rabid raccoons in Lake County and 2 adjacent counties (Geauga and Cuyahoga) (Figures 1 and 2, panel A). These detections raised the question whether current ORV and surveillance strategies are sufficient for containment and reaching the long-term goal of regional elimination of RRV. We used molecular analyses to gain insight into the factors and possible raccoon source populations associated with the breach of the ORV zone in Ohio.

The Study

Viral RNA was extracted as described (*6*) from brain tissue of 67 rabid raccoons. Samples came from raccoons in Ohio (1996 [n = 9] and 2004 [n = 10] outbreaks) and the neighboring states of Pennsylvania (2003–2004 [n = 21] and West Virginia (1987–2004 [n = 27]) (online Appendix Table, available from www.cdc.gov/EID/content/14/4/650-appT.htm). We amplified a 1,345-nt portion of the glycoprotein gene (G) and, for a smaller subset of samples (n = 20), the complete nucleoprotein gene (N) (1,416 nt,) (see [6] for primers and conditions). Sequences from a Florida raccoon (G, U27216; N, U27220) were included as an outgroup. When sequence data for G and N had been obtained, sequences were concatenated. After alignment, appropriate evolutionary models (*7*) were found for phylogenetic estimation by using maximum-likelihood and Bayesian approaches (*8*,*9*). Maximum-likelihood trees were constructed by using heuristic searches, and node support was assessed with 1,000 bootstrap replicates under the distance criterion with maximum-likelihood model settings. Bayesian estimation was performed with 2 runs of 6 million samples each and a sampling frequency of 1,000; the first 1,000 samples were discarded as burn-in.

A Bayesian molecular clock–based method (*10*) was used to estimate when the 2004 RRV lineage had started to diversify. To estimate evolutionary rates, we included 3 raccoon rabies sequences isolated during the larger Atlantic coast epizootic of 1982–1984. Analyses were run for 10 million steps after a burn-in period of 1 million under an exponential growth model; alternative demographic models produced equivalent results (data not shown).

According to the combined G and N data, the phylogenetic analyses showed that the 2004 Ohio outbreak was caused by a distinct RRV lineage that had limited diversity (Figure 2, panel B, red), which suggests a single-source introduction into Ohio. The 2004 lineage was not a direct descendent of any previously sampled lineages, but it shared a common ancestor with another lineage (Figure 2, panel B, yellow) that contained the viruses responsible for the 1996 Ohio outbreak along with contemporary viruses from western Pennsylvania. No members of either of these lineages had been found east of the ORV barrier (Figure 2, panel A), an area dominated by a different group of viruses (Fig-

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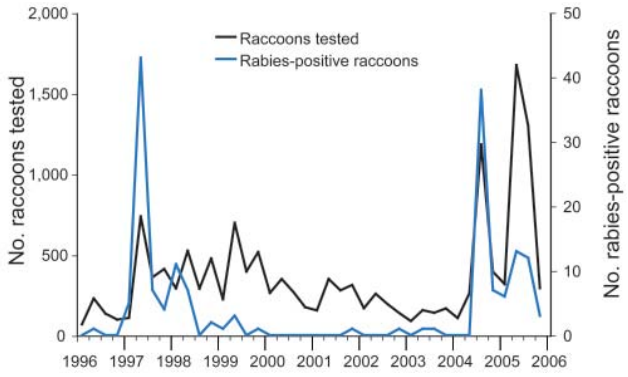

Figure 1. Raccoon rabies surveillance efforts in Ohio, 1996–2005. Data were aggregated at 3-month intervals.

ure 2, panel B, blue). The same result was obtained when the larger dataset based on G data only was analyzed (online Appendix Figure, available from www.cdc.gov/EID/content/14/4/650-appG.htm) and when we included RRV sequences from throughout eastern North America (data not shown). This finding suggests that the virus associated with the 2004 outbreak in Ohio most likely originated within the ORV zone.

Temporal estimates further indicated that all viruses sampled in the recent Ohio outbreak had started to diversify at least 3 years before 2004. The estimated dates associated with the most recent common ancestor were 1998 (highest posterior density interval 1993–2001) for the concatenated G and N data and 1995 (highest posterior density interval 1990–2000) for G data only.

Conclusions

Our findings imply that RRV had been circulating undetected among raccoons in the ORV zone, and possibly beyond it, for several years before its detection in 2004. These findings have important implications for the control of wildlife rabies in raccoons through ORV. First, the genetic analyses do not point to a long-distance transmission event to Ohio but rather suggest that the virus was indigenous to the region. In view of potential continued transmission events within the current ORV zone, widening the ORV corridor likely will not prevent such transmission and further spread. Second, the findings suggest that RRV may be able to persist within the ORV zone for several years and thus provide continued risk for eventual spread into unvaccinated raccoon populations. Insufficient levels of immunization among the overall population could contribute to this situation. However, spatial variation in the level of immunization or random fluctuations in the number of infected animals may also enable the virus to persist in parts of the ORV zone. Third, the level of surveillance needed to detect RRV when transmission frequency is low is unclear.

Our results indicate that the virus had been present within Ohio for several years when surveillance efforts were relatively low; from January 2000 through June 2004, an average of 71 raccoons were tested each month compared with an average of 139 per month during 1997–1999 (Figure 1). Therefore, the critical question is: at what point would the marginal cost of increased surveillance leading to earlier detection have outweighed the cost associated with controlling the 2004 outbreak? To develop the most cost-effective strategy for containment and ultimate elimi-

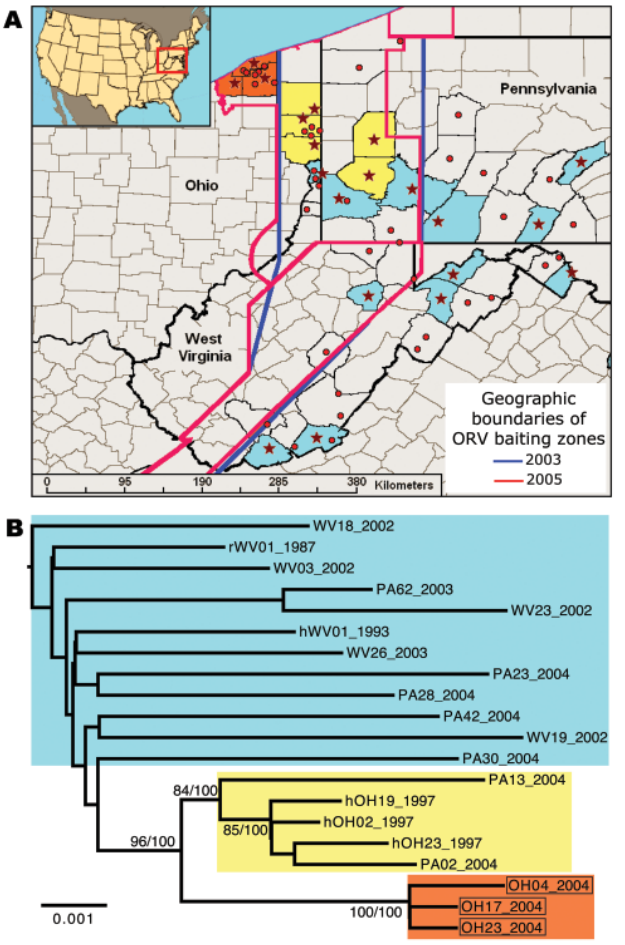

Figure 2. Spatial and genetic distribution of sequences of the raccoon rabies virus variant (RRV) from the 2004 Ohio outbreak relative to virus found in neighboring areas. A) Distribution of RRV samples included in phylogenetic analysis of G and N gene sequences (stars) or G sequences only (circles). Colors reflect phylogenetic groups as shown in panel B. B) Maximum-likelihood tree of concatenated G and N gene sequences of RRV sampled in or near Ohio, 1987–2004. Samples from the 2004 outbreak are boxed. Bootstrap values and corresponding Bayesian posterior values (% for both) are shown for key nodes. Tree was rooted by using RRV G and N sequences from a Florida raccoon (not shown). ORV, oral rabies vaccine. Scale bar = nucleotide substitutions per site.

nation of rabies among raccoons, further analyses should aim at quantifying this trade-off.

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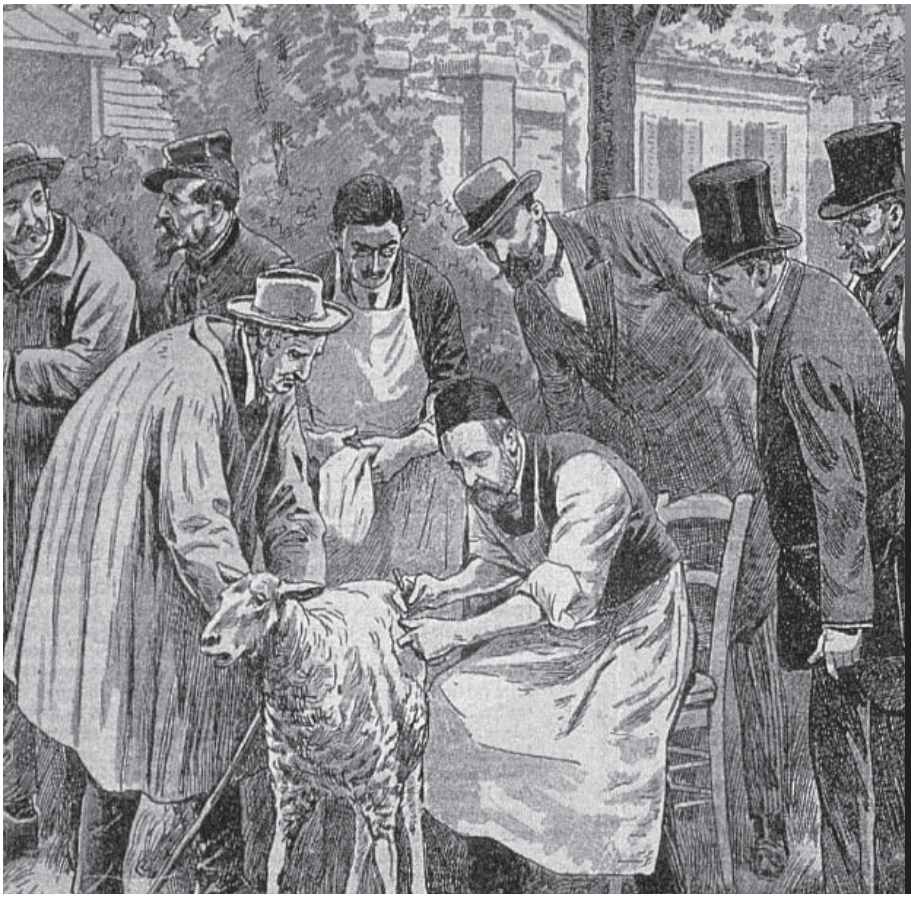

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Single Nucleotide Polymorphism Typing of *Bacillus anthracis* from Sverdlovsk Tissue

Richard T. Okinaka,*† Melinda Henrie,* Karen K. Hill,* KristinS. Lowery,‡ Matthew Van Ert,† Talima Pearson,† James Schupp,† Leo Kenefic,† Jodi Beaudry,† Steven A. Hofstadler,‡ Paul J. Jackson,*§ and Paul Keim*†¶

A small number of conserved canonical single nucleotide polymorphisms (canSNP) that define major phylogenetic branches for *Bacillus anthracis* were used to place a Sverdlovsk patient's *B. anthracis* genotype into 1 of 12 subgroups. Reconstruction of the *pagA* gene also showed a unique SNP that defines a new lineage for *B. anthracis*.

The 1979 accidental release of *Bacillus anthracis* spores in the northeastern quadrant of Sverdlovsk (today's Ekaterinburg) in the former Soviet Union resulted in a relatively large-scale incident of inhalation anthrax (*1*). Histologic examinations of formalin-fixed, paraffin-embedded tissue samples from patients affected by this incident showed pathologic changes (*2*). Paraffin-embedded material from 11 of these patients was also subjected to molecular DNA analysis to demonstrate the presence of *B. anthracis* plasmid markers (*3*). These tissue samples appeared to contain 3–4 allelic variants of *B. anthracis*, based on a single variable-number tandem repeat (VNTR) marker (*vrrA*), which suggests that the material contained multiple strains of this species (*4*).

An early global comparison of the entire *pagA* gene sequence from 26 diverse *B. anthracis* isolates and a single nucleotide polymorphism (SNP)–rich region (307 bp) of the samples from Sverdlovsk found 7 SNPs in this gene (*5*). Two of these SNPs were unique to 3 of the Sverdlovsk samples. However, the distribution of the remaining 5 SNPs separated the 26 diverse isolates and the remaining Sverdlovsk samples into clusters that were consistent with diversity groups previously described by amplified fragment–length polymorphism (AFLP) analysis of a larger subset of isolates (*6*). The AFLP analysis had separated a collection of 78 isolates into 5 diversity groups. Although only 307 bp of the Sverdlovsk tissue samples were sequenced, a single SNP *(pagA* SNP 3602) placed 7 of 10 Sverdlovsk tissue samples into a large diversity group called western North America (WNA) (*5*).

Recent comparisons among 5 *B. anthracis* whole genome sequences uncovered ≈3,000 SNPs, and the rigorous examination of ≈1,000 of these SNPs across 27 diverse isolates demonstrated an extremely conserved clonal population structure for this species (*7*,*8*). These results led to a genotyping method that uses a small number of canonical SNPs (canSNPs) to replace the analysis of ≈1,000 SNPs and still precisely defines key positions and branch points within the *B. anthracis* tree (*9*). This canSNP model was recently tested against a large, diverse *B. anthracis* collection of 1,033 isolates (*10*). When all 1,033 isolates were evaluated against a panel of 13 canSNPs, the results demonstrated that each *B. anthracis* isolate had 1 of only 12 different canSNP profiles. These analyses supported the notion that a single canSNP can be used to represent an entire genome when the genome being examined is as conserved as that of *B. anthracis* (*7–9*). This strategy was applied to the analysis of a Sverdlovsk sample that appears to represent a single diversity group previously recognized as WNA from *pagA* sequence analysis.

The Study

DNA was extracted by using QiaAmp DNA Mini Kits (QIAGEN, Valencia, CA, USA), following protocols recommended for formalin-fixed tissue samples. A Genomeplex Whole Genome Amplication Kit (Sigma, St. Louis, MO, USA) was used to preamplify the extracted DNA sample. Short DNA fragments were amplified from these DNA samples by using the following primer sets: canSNP.A.Br.008 F: 5′-GCCAAGATATTCGTGACATT-3′; canSNP.A.Br.008 R: 5′-TTTGGACCAGGTTTCTGTATTT-3′; canSNP A.Br. 009 F: 5′-TGCGGAATATCGTTAAGTAAT-3′; canSNP A.Br.009R: 5′-TGGACGTGAATTAGGAAAAGT-3′ (for traditional PCR and sequencing); canSNP A.Br.008 F: 5′-TCTAAGAAAGATTCGCAACTACGCTATAC-3′; canSNP A.Br.008 R: 5′-TGCATTCGCAACTACGCTATA CGTTTTAGATG-3′; canSNP A.Br.009 F: 5′-TGCC GGGGTTTCTACTGTGTATGTTGT-3′; canSNP A.Br. 009 R: 5′-TGGGTTAGGTATATTAACTGCGGATG ATGC-3′ (for mass spectrometry base composition analysis); *pagA* 981 F: 5′-AATGAGGATCAATCCA CACAG-3′; and *pagA* 981 R: 5′-ATTTAAACCCA TTGTTTCAGC-3′. Traditional PCR amplification schemes and DNA sequencing techniques were used for sequencing with an ABI 3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The mass spectrometry resequencing technique is described in detail elsewhere (*11*).

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Primers for real-time PCR were as follows: PagSNP.981F 5′-CACAGAATACTGATAGTCAAACGAGAACA-3′; Pag SNP.981R 5′-GCACTTCTGCATTTCCATGTACTT-3′; probe 1: PagSNP.981T VIC 5′-ACAAGTAGGACt CATAC-3′; and probe 2: PagSNP.981A 6FAM 5′-CAAG TAGGACaCATACT-3′.

The Figure illustrates the relative positions of 3 canSNPs along the branches of the canSNP-derived phylogenetic tree for the 1,033 *B. anthracis* isolates (*10*). The focus of this diagram is the WNA lineage (A.Br.WNA), which forms a branch and a node part way along this branch. CanSNP typing both by traditional sequencing and by the mass spectrometry resequencing technique of the 2 positions (*10*) that define the WNA lineage (A.Br.WNA) and a branch point designated A.Br.008/009 (canSNPs in red; A.Br.008, and A.Br.009, respectively; Figure) confirms that the Sverdlovsk sample 7.RA93.15.15 (Table) is related to the WNA lineage. However, the canSNP genotype of the Sverdlovsk sample indicates that although it may share a common ancestor with the WNA lineage, it is a member of the A Branch.008/009 subgroup that comprises isolates (n = 154) that were recovered primarily from Europe and portions of Asia (*10*). The close relationship between the WNA sublineage and this major European/Asian subgroup is consistent with the hypothesis that *B. anthracis* was introduced into the North American continent by European settlers, possibly from France or Spain (*10*,*12*).

The early *vrrA* and *pagA* gene studies on Sverdlovsk tissue samples suggested that they contained multiple strains of *B. anthracis* because several *vrrA* alleles and *pagA* genotypes were noted. However, a dominant *vrrA* allele (4 repeats), as measured by both the quantity and the quality of the PCR products, was found in 9 of 13 samples from the tissue specimens (*3*). In some samples, it was the only allele present. Similarly, the *pagA* diversity group V signal (*pagA* SNP 5 at bp 3602 or bp 1791 from the ATG start) for the WNA subgroup appeared in 7 of 10 samples of the Sverdlovsk tissues (*5*). Sverdlovsk sample 7.RA93.15.15 contained these dominant allele signals, which suggests that these signals represent a single, specific strain of this pathogen.

In addition to the analysis of the canSNPs that define the A.Br.008/009 sublineage, large portions of the *pagA* gene sequence were reconstructed from sample 7.RA93.15.15 by using 40 overlapping PCR fragments without whole genome amplification. The reconstruction of this gene as well as other loci was not always feasible for many of the remaining Sverdlovsk samples because of limited DNA. Sequence analysis of these PCR products showed a new A–T synonymous transversion mutation (Table) at *pagA* position bp 981, where the start ATG = bp 1, 2, 3, or position 2784 by using the coordinates described by Price et al. (*5*). This SNP was not found in any of the original 27 diverse *B. anthracis* isolates (*5*) or in a recent *pagA* gene sequencing analysis of 124 archival *B. anthracis* samples from the Centers for Disease Control and Prevention (*13*). The status of the new *pagA* 981 site was also analyzed in a panel of isolates containing 89 diverse multiple-locus, variable-number, tandem-repeat analysis genotypes and an additional 154 A.Br.008/009 subgroup (Eurasian) isolates by using a custom made real-time PCR assay: TaqMan Minor Groove Binding probes and primers for SNPs (Applied Biosystems) (*14*). These analyses indicate that the *pagA* 981 A–T transversion represents a rare SNP allele found in only 3 Eurasian isolates and the Sverdlovsk 7.RA93.15.15 sample. (The Table shows isolates with the rare allele and a sampling of the additional Eurasian isolates.) This real-time PCR protocol is applicable to low copy–number templates and, somewhat, to mixed sample analyses. We applied it to

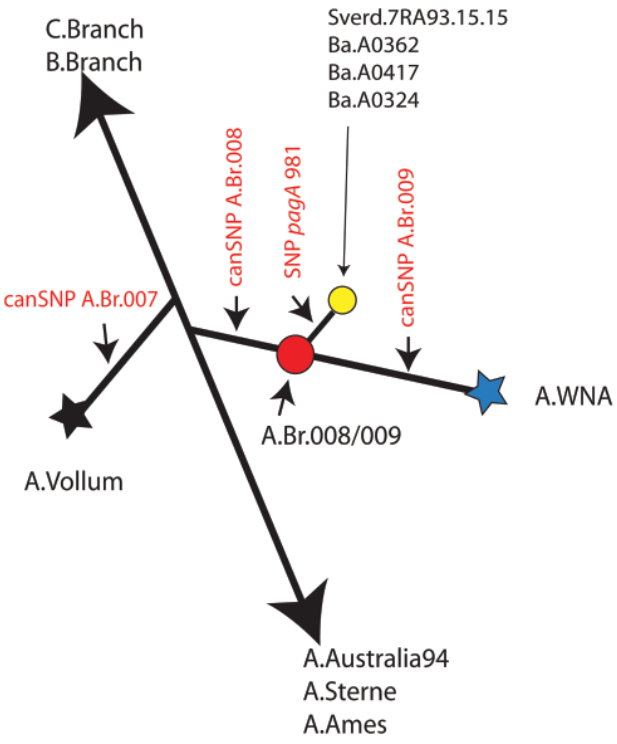

Figure. Schematic single nucleotide polymorphism (SNP) tree for *Bacillus anthracis.* This tree illustrates the relative positions of several sequenced strains of *B. anthracis* that form the specific sublineages in the A group of *B. anthracis* and in particular the Western North American lineage (A.BR.WNA, represented by a blue star [*10*]). The canonical SNPs and their positions are depicted in red lettering. A branch point (red circle) or node designated A.Br.008/009 originally represented 154 isolates and canSNP analysis places Sverdlovsk 7.RA93.15.15 in this node. The new *pagA* SNP981 defines a new branch radiating from this node and contains at least 3 other isolates.

Table. Canonical single nucleotide polymorphism (canSNP) typing and a new *pagA* SNP from Sverdlovsk 7.RA93.15.15 (spleen)*

Isolate	Country of origin	Lineage/group (*10*)	canSNP, A.Br.007	canSNP, A.Br.008	canSNP, A.Br.009	SNP, *pagA* 981
A1055		C.Br.A055	T	T	A	–pXO1
KrugerB		B.Br.KrugerB	T	T	A	A
CNEVA.9066		B.Br.CNEVA	T	T	A	A
Ames		A.Br.Ames	T	T	A	A
Sterne		A.Br.Ames	T	T	A	A
Australia94		A.Br.Aust94	T	T	A	A
Vollum		A.Br.Vollum	C	T	A	A
(Branch point)		A.Br.008/009	T	G	A	
Sv7,RA93.15.15 (spleen)		A.Br.008/009	T	G	A	T
Sv31.RA93.39.3						T
Sv40.RA93.40.5						T
Sv25.RA93.031						T
Sv1.RA93.42.1						T
Sv33.RA93.20.5						T
Sv21.RA93.38.4						T
Western North America		A.Br.WNA	T	G	G	A
A0362	Norway	A.Br.008/009	T	G	A	T
A0417	Hungary	A.Br.008/009	T	G	A	T
A0324	Slovakia	A.Br.008/009	T	G	A	T
A0293	Italy	A.Br.008/009	T	G	A	A
A0463	Pakistan	A.Br.008/009	T	G	A	A
A0149	Turkey	A.Br.008/009	T	G	A	A
A0264	Turkey	A.Br.008/009	T	G	A	A
A0032	China	A.Br.008/009	T	G	A	A
A0033	China	A.Br.008/009	T	G	A	A
A0241	Turkey	A.Br.008/009	T	G	A	A
A0245	Turkey	A.Br.008/009	T	G	A	A
A0463	Pakistan	A.Br.008/009	T	G	A	A

*This table illustrates 2 canSNPs that distinguish the subgroup/branch point A.Br.008/009 from the Western North American (WNA) sublineage. The table also depicts the status of the pagA 981 SNP in 7 of 12 Sverdlovsk samples and several other key isolates. Black shading, WNA lineage; gray shading, subgroup A.Br.008/009.

DNA extracted from the remaining 12 Sverdlovsk tissue samples (*3*). Seven of these templates supported extensive amplification and detected only the "T" allele at this SNP locus (Table) with no evidence for the alternate "A" allele.

The tissue samples with *pagA* 981 SNP real-time PCR amplification were the same as samples that displayed the single *vrrA* 4-repeat signal (group V), except for 1 sample (40.RA93.40.5, spleen) that contained a mixture of the *vrrA* 2, 4, and 5 repeats. With this exception, a mixed signal could not be predicted from the previous results (*3*,*5*). Why a mixed signal was not seen in the latter sample is not clear, although multiple explanations exist, including the loss or curing of plasmid in certain genomes during mass culture. The remaining samples with multiple *vrrA* alleles did not produce amplicons in the *pagA* 981 real time assay.

Conclusions

CanSNP typing provides insight into a dominant strain involved in the 1979 Sverdlovsk anthrax outbreak and identifies it as part of a large cluster of isolates (A.Br.008/009) that are found in Europe and Asia. Our findings are consistent with claims that the weaponized strain released in Sverdlovsk (Anthrax 836) was originally isolated from a rodent in the city of Kirov, Soviet Union, in the mid-1950s (*15*). These findings are not in conflict with reports that grouped the Sverdlovsk strain with the WNA sublineage because this recently defined Eurasian cluster shares a common ancestor with the WNA isolates (*7*,*10*). Reconstruction of the *pagA* gene sequence from this Sverdlovsk sample uncovered a new SNP at position 981, which appears to be specific for a small subset of Eurasian isolates. This SNP creates a branch emanating from the A.Br.008/009 node and currently contains only 3 isolates plus the prominent Sverdlovsk genotype. The extremely conserved nature of the *B. anthracis* genome (*7*), coupled with analysis of more than 230 close and diverse isolates, suggests that the *pagA* 981 transversion represents a new canSNP that can rapidly identify the closest relatives of this distinct Sverdlovsk lineage.

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Dr Okinaka is a research scientist at Northern Arizona University. His interests have focused on the molecular analysis of mutations in humans and microbial pathogens, with particular emphasis on the sequencing analysis of *B. anthracis* and its plasmids.

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Human *Mycobacterium bovis* Infection and Bovine Tuberculosis Outbreak, Michigan, 1994–2007

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Mycobacterium bovis is endemic in Michigan's white-tailed deer and has been circulating since 1994. The strain circulating in deer has remained genotypically consistent and was recently detected in 2 humans. We summarize the investigation of these cases and confirm that recreational exposure to deer is a risk for infection in humans.

Historically, *Mycobacterium bovis* infection in humans was associated with consumption of unpasteurized milk and dairy products (*1*,*2*) and this is still the most important route of exposure in developing countries. US populations are exposed to unpasteurized dairy products imported from countries where *M. bovis* is prevalent (*3*,*4*). *M. bovis* infection in humans is of concern to health officials in Michigan because of to its endemicity in the state's wild white-tailed deer population and its discovery in several cattle herds. *M. bovis* in deer represents possible occupational and recreational routes of exposure to humans, especially for hunters, trappers, taxidermists, venison processors, and venison consumers (*5*).

Although *M. bovis* is a zoonotic agent, surveillance indicates no increase in its incidence in Michigan residents since an outbreak began in 1994. Since 1995, the incidence rate of *M. bovis* infection in Michigan residents has remained very low, with ≈1 new case per year for a total of 13. No genetic or epidemiologic link to the deer/cattle outbreak strain has been identified among 11 of these human *M. bovis* cases, based on restriction fragment-length polymorphism analysis, spoligotyping, or mycobacterial interspersed repeat units (MIRU) typing (M. Wilkins, unpub. data, Michigan Department of Community Health, March 2007). Table 1 shows the spoligotyping and MIRU typing results from 9 available human specimens that were unrelated to the deer/cattle outbreak strain. All genotyping of isolates mentioned in this report was performed at the Michigan Department of Community Health, Bureau of Laboratories, Lansing, MI, USA, by using currently recommended guidelines (Centers for Disease Control and Prevention, Atlanta, GA, USA) (*8*–*10*). The remaining 2 human cases of *M. bovis* occurred in US-born, Michigan residents; the cases had epidemiologic and molecular links to the genotypically consistent deer/cattle outbreak strain circulating in Michigan.

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The Cases

Patient 1, 2002

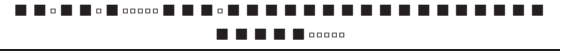
In January 2002, a 74-year-old man sought medical care, reporting malaise, anorexia, and fever. Past medical history included ischemic bowel disease, vascular disease, partial gastrectomy for peptic ulcers, and left upper lobectomy for squamous cell carcinoma (December 1999). On February 1, he was hospitalized with persistent fever and nonproductive cough; results of a chest radiograph were consistent with necrotizing pneumonia. A tuberculosis (TB) skin test (TST) result was negative, and a sputum smear was negative for acid-fast bacilli (AFB). After 5 days, the patient had not improved clinically; chest radiograph showed increasing infiltrate on the left side. Diagnostic bronchoscopy was performed, which yielded an AFB-positive smear. The condition of the patient deteriorated clinically over the next 10 days; he died on day 16 of his hospitalization.

Laboratory confirmation for TB, speciation, and antimicrobial drug susceptibility testing were pending at the time of his death. Genotyping analysis showed that the *M. bovis* isolated from this patient matched the circulating deer/cattle strain (Table 2).

In his youth, patient 1 lived on a farm geographically distant from the current bovine TB–endemic area. His first wife had a reported diagnosis of TB after their divorce >40 years before, and his second wife reports he drank unpasteurized milk as a youth. He moved to the edge of Deer Management Unit (DMU) 452 in 1994, which is the focal area for the bovine TB outbreak in deer. There, he ran a business with a buck pole where hunters displayed killed deer. Additional potential exposures included hunting white-tailed deer and consuming venison (>10 years before his death), handling a deer carcass from the DMU 452 vicinity in 2000, and recreational feeding of deer.

This patient was in poor health at the time of death, having acute and chronic illness. His poor health would

Table 1. Nonepidemiologically linked human *Mycobacterium bovis* spoligotyping and MIRU typing results for 9 available human specimens, Michigan*

Country of birth	Site	Year collected	International spoligotype†	MIRU type	Spoligotypes
USA	Cervical LN	1997	SB1210	Not available	
USA	Spine	1997	SB0131	Not available	
Mexico	Abd abscess	1998	SB0140	232224152322	
Mexico	Sputum	1998	SB1210	Not available	
Bosnia	Cervical LN	2000	SB1215	232314353312	
Mexico	Sputum	2003	SB0121	232314223322	
USA	Cervical LN	2003	SB0121	232324252322	
Mexico	Cervical LN	2005	SB1216	232124233222	
Mexico	Cervical LN	2007	SB0140	231224243322	

*MIRU, mycobacterial interspersed repeat units (*6*); LN, lymph node; Abd, abdominal.
†International Spoligotype Database, available from www.mbovis.org (*7*).

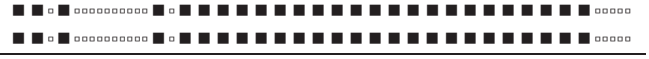
have rendered him more susceptible to infection with *M. bovis* and would have made the progression from latent infection to clinical disease more likely. The pathology results from his lung resection in December 1999 provided no evidence of TB; therefore, infection was likely acquired subsequently. The genotyping results from patient 1 matched those of the circulating deer/cattle strain, which suggested exposure to infected cattle or deer. The lack of recent exposure to cattle suggests that deer are the more likely source of infection.

Patient 2, 2004

Patient 2 was a 29-year-old, previously healthy man. On October 1, 2004, he shot a white-tailed deer just outside DMU 452. While field dressing the animal, he punctured his left index finger with a hunting knife. Approximately 18 days after the injury, his finger became inflamed and painful, so he sought medical treatment. Based on his history of exposure to a deer with lesions, a TST was administered; the result was negative. After ≈10 days of antimicrobial drug therapy, the wound had not improved. He was hospitalized, and the infected finger was incised and drained. An orthopedic specialist diagnosed infectious tenosynovitis of the flexor tendon of the left index finger. The initial slide preparation was negative for AFB. A wound culture was sent to Michigan Department of Community Health, Bureau of Laboratories.

The patient was discharged and then readmitted to the hospital 12 days later with subcutaneous infection at the puncture site, which was again incised and drained. A slide made of growth from the broth culture medium was positive for AFB. Genetic probe results confirmed *M. tuberculosis* complex. By December 7, 2004, the culture was reported as resistant to pyrazinamide, suggesting *M. bovis,* which was later confirmed on the basis of susceptibility to thiopene-2-carboxylic acid hydrazide and biochemical testing for pyrazinamidase. The result of a second skin test, 14 weeks postexposure, was positive (6-mm induration).

Table 2. Epidemiologically linked *Mycobacterium bovis* spoligotyping and MIRU typing results, Michigan*

Species	Year collected	International spoligotype†	MIRU type	Spoligotypes
Deer 1	1997	SB0145	Not available	
Bovine 1	1998	SB0145	Not available	
Bovine 2	1999	SB0145	232224253322	
Bovine 3	1999	SB0145	232224253322	
Bovine 4	2002	SB0145	232224253322	
Human patient 1	2002	SB0145	232224253322	
Deer 2	2003	SB0145	232224253322	
Human patient 2	2004	SB0145	232224253322	
Deer 3‡	2004	SB0145	232224253322	

*MIRU, mycobacterial interspersed repeat units (*6*).
†International Spoligotype Database, available from www.mbovis.org (*7*).
‡Deer shot by patient 2.

He continued to receive antimicrobial drug therapy for 9 months without further complications

As an experienced hunter, patient 2 recognized the tan nodules in the deer's chest cavity as *M. bovis* and promptly buried the carcass. In December, he led Michigan Department of Natural Resources staff back to the carcass, which was retrieved; the chest cavity was filled with lesions (Figure). Although the carcass was buried for >9 weeks, chest cavity samples were submitted for culture. After numerous attempts with alternative decontamination techniques, a viable culture was obtained. Genotyping results of the carcass isolate were identical to that recovered from patient 2 and the circulating deer/cattle strain (Table 2). The investigation of the infection in patient 2 provided strong evidence of transmission of *M. bovis* infection from deer to human through percutaneous injection with a contaminated hunting knife. The patient's history of hunting exposure was essential to diagnosis and treatment of this rare form of TB.

Conclusions

Although epidemiologic evidence presented for patient 1 is not irrefutable, we conclude that both cases are part of a cluster that is epidemiologically and genotypically confirmed (*11*). The initial TST result was negative in both of these cases, likely due to cutaneous anergy (patient 1) and administration too soon after exposure (patient 2). Initial negative skin test results made diagnosis problematic for healthcare providers.

The confirmation of a hunter's acquiring cutaneous *M. bovis* from an infected deer supports the need for public health precautions. First, hunters should wear heavy latex or rubber gloves while field dressing deer. Second, hunter education was important in the second case because the hunter recognized the deer as infected and specifically mentioned his exposure each time he sought medical treatment. Third, efforts to raise the index of suspicion of the medical community regarding cutaneous and other occupational or recreational exposures to TB continues to be important, so that appropriate diagnoses can be made. Finally, in both cases, the initially negative TST result complicated the diagnostic efforts. It is an ongoing challenge to ensure that providers appropriately apply and interpret the TST.

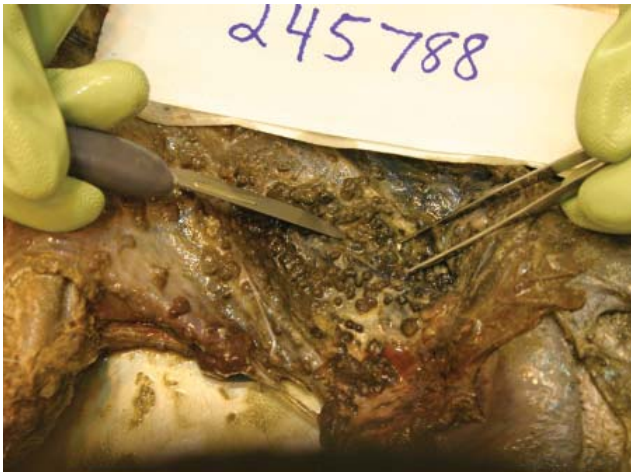


Figure. Chest cavity of a deer shot by patient 2; the deer was retrieved after being buried for 9 weeks. The photo shows the classic nodular lesions of *Mycobacterium bovis* infection. Photo: J.S. Fierke, D.J. O'Brien, S.M. Schmitt, Wildlife Disease Laboratory, Michigan Department of Natural Resources.

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Mycobacterium avium Lymphadenopathy among Children, Sweden

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We studied *Mycobacterium avium* lymphadenopathy in 183 Swedish children (<7 years of age) from 1998 through 2003. Seasonal variation in the frequency of the disease, with a peak in October and a low point in April, suggests cyclic environmental factors. We also found a higher incidence of the disease in children who live close to water.

Mycobacterium avium is the most common of the nontuberculous mycobacteria that infect otherwise healthy children (*1*). It manifests as a chronic granulomatous lymphadenopathy in the neck region that preferably is treated by excision of the affected lymph node (*2*). In Sweden the incidence of nontuberculous mycobacterial disease in children increased from 0.06/100,000 population to 5.7/100,000 after discontinuation of the general BCG (Bacillus Calmette-Guérin) vaccination program in 1975 (*3*).

M. avium is common in the environment. It has been isolated from natural and human-made water systems as well as from soil and animals (*4*,*5*). The role of animals in its epidemiology is not clear. The main hypothesis is that oral contact with *M. avium*–infected water causes lymphadenitis in the head and neck region (*4*). An environmental study in nearby Finland found nontuberculous mycobacteria in 100% of brook water samples (*6*).

Human populations who live close to water have a higher proportion of persons with positive reactions to *M. avium* and other nontuberculous mycobacterial sensitins (*7*,*8*). In Sweden, 25.4% of non-BCG–vaccinated schoolchildren in an urban coastal area reacted to *M. avium* sensitin (*7*). In studies from Canada and the United States/Canada, *M. avium* disease in children has previously shown no seasonal variation (*9*), or it has been dominant in winter, spring, or both (*1*,*2*).

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In Sweden, nontuberculous mycobacterial infection has been a reportable disease since 1989; all cases are reported to the Swedish Institute for Infectious Disease Control. From 1998 through 2003, 186 culture-positive cases of *M. avium* infection in children <7 years of age were reported. We registered age and residence and reviewed the children's medical records to establish the month of onset of disease (date parent discovered an enlarged lymph node). Permission to conduct the study was obtained from 6 of the 7 ethical committees in Sweden. We collected information from 127 patient records. To these we added 35 cases that occurred during 1983–1997 (*3*,*10*). One patient with lung infection and 2 children whose home addresses could not be located were excluded. Altogether, 183 children (1998–2003) were included in the geographic analysis, and 162 children (1983–2003) with culture-proven *M. avium* lymphadenopathy were included in the analysis of seasonal variation.

Sweden is divided into 290 urban and rural districts. The exact number of children <1–6 years of age in every district is known for each year during 1998–2003. Each district was evaluated by using a map with a scale of 1:300,000. Within each district we estimated the proportion of the total child population 1) living within 5 km of the coast or a big lake or within 2 km of a small lake or river and 2) living in the different cultivation zones 1–8 (zone 1 being the warmest, as defined by the Swedish association for leisure gardeners). A cultivation zone and a water category (salt or brackish water, fresh water, or no water) were assigned to each case-patient according to the home addresses of the children. We assumed most children were infected in the area where they resided.

Incidence rates were calculated and the corresponding 95% confidence intervals estimated by using the Poisson distribution, since events that occur randomly in time follow this distribution. Results of seasonal variations were tested with a commercial statistical software (Statistica version 7.1; Statsoft, Tulsa, OK, USA), by using nonlinear regression fitting a sine function (Figure 1).We considered p values <0.05 statistically significant. (See also online Appendix Table, available from www.cdc.gov/EID/content/14/4/661-appT.htm.)

The overall annual incidence rate of culture-verified *M. avium* lymphadenopathy in children <1–6 years of age in Sweden during 1998–2003 was 4.5/100,000 children. Seasonal variation was statistically significant ($p<0.05$), with a peak in October and a low point in April (Figure 1). The mean time from onset of symptoms at home to diagnostic puncture at a hospital was 4.3 weeks. The incubation period is unknown. From in vitro growth data, we assumed that the time of infection was 2–8 weeks or longer before clinical manifestations. Seasonal variation tended to be more accentuated in children ≤24 months old (Figure

1). Our interpretation is that younger children might have shorter incubation periods (weeks) because of their immature immune systems (*11*), whereas the incubation periods of older children might be longer (weeks to months) and more variable, resulting in a shift to the right and a flattened sinusoidal curve. Thus, the curve of the data for the younger children would be closer to an imagined curve of "true" inoculation time.

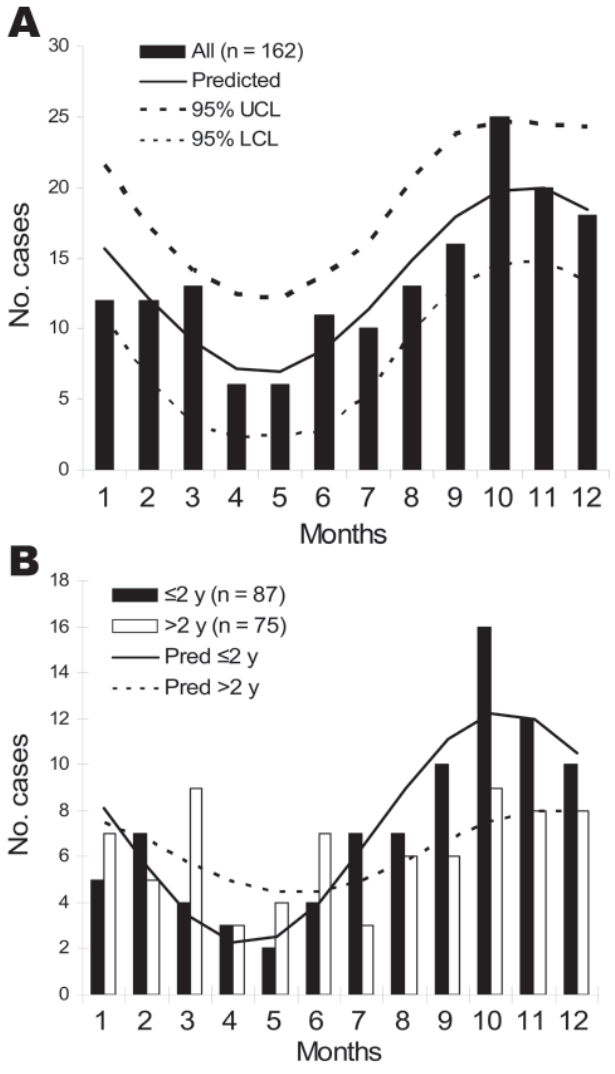
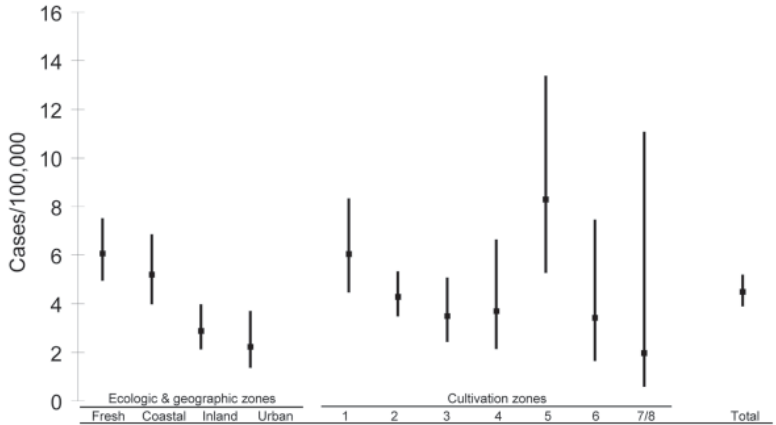


Figure 1. Seasonal incidence of *Mycobacterium avium* infection in Swedish children (1983–2003) in our study (bars = real numbers) and as predicted by nonlinear regression sine functions (equations: $y = a + b\sin[(x - c)\Pi/6]$, where x represents the months (1–12) (www.smhi.se), and with "a," "b," and "c" characteristic for each curve and $b \neq 0$ with statistical significance, $p<0.05$, for all these curves. (See also online Appendix Table, available from www.cdc.gov/EID/content/14/4/661-appT.htm.) A) All children. The curves were statistically significant, $p<0.05$, for both 1983–1997 and 1998–2003, and so the data for all years were grouped together. UCL, upper confidence limit; LCL, lower confidence limit. B) Children ≤2 years and >2 years of age, respectively. "b," amplitude of curve, has a tendency to be greater for children ≤2 years of age ($p = 0.07$) and "c" is slightly smaller for children >2 years of age, representing a shift to the right of the curve, though not statistically significant. Pred, predicted.

We found no significant difference in seasonal variation when we compared colder (*4*–*7*) and warmer (*1*–*3*) cultivation zones. Because of compensation after the vernal equinox of more light in the North, the beginning of meteorologic spring and summer differs no more than 1 month between cultivation zones (www.smhi.se). Spring means better temperature and nutritional conditions for growth of bacteria in nature. *M. avium* can enter a metabolic state of dormancy in response to starvation and recover rapidly when conditions improve again (*12*). We speculate that the observed seasonal variation is due to a combination of changing temperature and nutritional conditions in the environment throughout the year as well as changing human activities in different seasons. The reason why previous studies have not shown this seasonal variation might be due to a smaller number of investigated cases (*9*), the inclusion of more heterogeneous materials (older children and other mycobacteria beside *M. avium*), or conduction of studies in regions with less clear-cut seasonal variations than Sweden.

Our results also show a correlation between *M. avium* clinical disease in children and living close to water (Figure 2). Socioeconomic status usually is associated with living close to water. In Sweden, however, socioeconomic differences in society are small and therefore not likely to be a confounding factor. Correlation to water has previously only been incidental—indirectly implied by studies of sensitin reactivity in healthy populations (*7*,*8*) and by the isolation of *M. avium* from natural and man-made water systems (*4*). However, 1 Swedish sensitin study, conducted in an urban area situated by the sea, disagrees with our results. It found a high sensitivity to *M. avium* PPD (*7*), whereas the incidence of clinical disease in our material is low in the same area. We speculate that the reason for this could be that inhalation of *M. avium*–containing aerosols might be sufficient to convey sensitin positivity but that acquisition of the clinical disease demands close contact with and oral ingestion of natural water, which are less likely to happen to a child who lives in a city. Seasonal variation and higher incidence in regions near natural fresh water compared to inland areas and cities indicate that the source of *M. avium* transmission is outdoor natural water rather than tap water.

We found an extremely high incidence of *M. avium* lymphadenopathy in a few contiguous districts of cultivation zone 5 in northern Sweden, where the human population has historically been isolated and where some genetic diseases exist (*13*). The higher frequency of *M. avium* lymphadenopathy in this exceptional area raises the possibility that development of clinical disease requires a geneti-



	Ecologic & geographic zones				Cultivation zones							
	Fresh	Coastal	Inland	Urban	1	2	3	4	5	6	7/8	Total
Cases	85	60	38	15	38	83	27	11	17	6	1	183
Incidence rates	6	5.2	2.8	2.2	6	4.2	3.4	3.6	8.3	3.4	1.9	4.4
95% UCL	7.5	6.8	3.9	3.7	8.3	5.3	5	6.6	13	7.4	11	5.1
95% LCL	4.9	3.9	2.1	1.3	4.4	3.4	2.4	2.1	5.2	1.6	0.5	3.8

Figure 2. Number of cases, incidence rates (cases/100,000 children/year), and 95% confidence intervals of *Mycobacterium avium* disease in children grouped according to ecologic, geographic, and cultivation zones, Sweden, 1998–2003. Freshwater, coastal (incidence of saltwater and brackish water were similar within this group), inland, urban (Stockholm, Göteborg, and Malmö, the 3 largest cities in Sweden) areas and the different cultivation zones (1–8, zone 1 being the warmest) are depicted. When assigning zones to each case, we assumed that the children were infected in the area where they resided. UCL, upper confidence limit; LCL, lower confidence limit.

cally predisposed host. Children with localized *M. avium* lymphadenopathy might not have the known and more severe impairment of γ-interferon–mediated immunity (*14*), but their immune status should be further investigated for milder defects.

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Kala-azar Epidemiology and Control, Southern Sudan

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Southern Sudan is one of the areas in eastern Africa most affected by visceral leishmaniasis (kala-azar), but lack of security and funds has hampered control. Since 2005, the return of stability has opened up new opportunities to expand existing interventions and introduce new ones.

Visceral leishmaniasis (kala-azar) is a deadly disease caused by the *Leishmania* protozoan parasite and transmitted through the bite of sandflies. Without prompt appropriate treatment, as many as 95% of kala-azar patients die, resulting in at least 50,000 deaths per year worldwide (*1*). Each death equates to a loss of 34 disability-adjusted life years (*2*). Continuous and large-scale control of kala-azar in the 2 foci of Southern Sudan has been hampered by war and instability. However, after the Comprehensive Peace Agreement between Northern and Southern Sudan was signed on January 9, 2005, the return of relative stability to Southern Sudan is now opening up new opportunities for supporting and improving healthcare delivery. To raise international awareness of kala-azar in Southern Sudan, we reviewed the available data and interventions and report the current status and plans for control of kala-azar.

The Review

Kala-azar occurs in 2 foci (Figure 1) and is caused by *L. donovani*. In the northern focus (Upper Nile, Jonglei, and Unity states), *Phlebotomus orientalis* is the vector; in the southern focus (parts of Eastern Equatoria state), *P. martini* is the vector (*3*,*4*). Although studies in eastern Sudan have found domestic animals infected with the parasite (*5*,*6*), whether these animals play a role as disease reservoirs has not yet been proven; thus, transmission is still thought to be anthroponotic.

The disease was first reported from Southern Sudan in 1904, and the first epidemic was documented in 1940 with a death rate of 80% (*7*). Beginning in 1984, an epidemic (unrecognized until 1988) devastated the western part of Upper Nile state, ultimately causing ≈100,000 deaths in a population of 280,000 over a 10-year period (*3*).

Passive case-detection data on kala-azar in Southern Sudan, collected by the World Health Organization (WHO) since 1989, indicate a cyclical pattern of kala-azar with considerable variation in the caseload from year to year (Figure 2). The dynamics presented in Figure 2 also suggest that Southern Sudan is currently between epidemics and provide a warning that cases may rise dramatically in coming years. In 2006, a total of 1,117 cases were reported, 65.4% of which were primary cases; the remainder were either relapses or cases of post–kala-azar dermal leishmaniasis. From January through June 2007, a total of 492 cases were reported, of which 88.2% were primary cases. The 5 locations accounting for 74.2% of the primary cases in 2007 were Malakal (n = 83), Ulang (n = 72), Nasir (n = 63), and Kiechkuon (n = 25) in Upper Nile state and Lankien (n = 79) in northern Jonglei state. Since 2002, the case-fatality rate recorded at healthcare facilities has been 4%–6%.

These data likely underestimate the actual number of cases because healthcare providers do not always provide complete reports and many kala-azar patients never visit healthcare facilities. Epidemiologic modeling of data from Upper Nile state estimated that those who visited healthcare facilities from October 1998 through May 2002 represented only 55% of cases and that 91% of kala-azar deaths were undetected (*8*). Health coverage is so minimal that some patients must walk for several days to access even the most basic healthcare services.

Figure 1. Map of Southern Sudan showing the 2 foci of visceral leishmaniasis. Shaded areas represent those counties where primary cases were reported from January through June 2007. Insert shows location of Sudan in Africa. (Adapted from World Health Organization, Southern Sudan Health Update, July–August 2007.)

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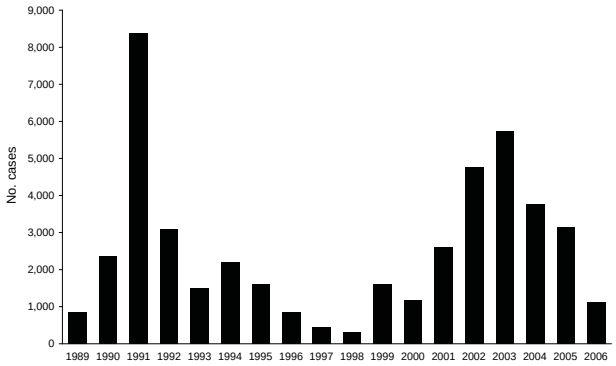

Figure 2. Total annual number of kala-azar cases in Southern Sudan reported to the World Health Organization, 1989–2006.

Despite the availability of different rapid diagnostic tests, most facilities used clinical diagnosis alone until recently. Only a few had the supplies and equipment to confirm suspected cases through microscopic examination of lymph node aspirate, which has a sensitivity of only 53%–65% (*1*). Nongovernment healthcare providers and Government of Southern Sudan–administered healthcare facilities thus had to confirm suspected kala-azar by direct agglutination test before they could receive a supply of the first-line treatment—sodium stibogluconate—from WHO through Pharmaciens sans Frontières. The use of the direct agglutination test was required because of concerns about the sensitivity and specificity of the rK39 dipsticks in East Africa; a recent study suggests that these concerns were well founded (*9*). Also, many facilities that had received dipsticks were not using them. Until 2004, many healthcare facilities did not have the equipment or skills to conduct direct agglutination tests, and blood samples had to be sent to Kenya for analysis, which often led to treatment delays as long as 18 days. Now some healthcare facilities can analyze these samples internally and start treatment within 24 hours.

Confirmed cases are treated with sodium stibogluconate at a dose of 20 mg/kg/day for 30 days. Recently, because of an increasing number of patients in Upper Nile state who were nonresponsive to sodium stibogluconate, Médicins sans Frontières tested a combination of sodium stibogluconate and paromomycin, which would reduce treatment duration (from 30 to 17 days) and cost. Patient survival and initial cure rates were better than those for patients who received sodium stibogluconate monotherapy (*10*). However, completion of multicountry phase III trials being conducted by the Drugs for Neglected Diseases Initiative (www.dndi.org) is eagerly awaited before the combination can be considered as an alternative. Amphotericin B, a second-line drug for treatment of kala-azar, is not yet available in Southern Sudan's facilities except in those run by Médicins sans Frontières.

Much remains unknown about the epidemiology of kala-azar in Southern Sudan (*11*). In the absence of detailed information on risk factors (cultural, demographic, epidemiologic, clinical, and geographic), use of long-lasting insecticide-treated nets seems a suitable method of prevention. Results from studies in North Sudan showed that insecticide-treated nets provided 27% protection from kala-azar (*12*). Whether similar protection can be achieved in Southern Sudan's disease-endemic areas requires confirmation because effectiveness is dependent on human and vector behavior (*13*).

The return of stability to Southern Sudan has opened up new challenges and opportunities for kala-azar control. Large-scale population movement of susceptible or infected populations into kala-azar–endemic or –nonendemic areas respectively, poses a major epidemic risk. The healthcare systems are weak and rely on support from faith-based and nongovernment organizations, which need to be coordinated to ensure consistency in diagnosis, treatment, and prevention. As health infrastructure and human resources are being built up, kala-azar will need to be addressed as an integral part of multifunctional healthcare delivery by government staff, but this requires training and the provision of essential supplies.

Kala-azar falls under the mandate of the Director General of Preventive Medicine within the Ministry of Health–Government of Southern Sudan. The Ministry of Health, with support from WHO and in conjunction with nongovernment organizations working on kala-azar, has embarked on a number of activities to strengthen case-management. Laboratory technicians in most referral facilities have now been trained on the direct agglutination test; case-management guidelines have been updated; the essential drugs list is being reviewed and expanded to include alternatives for second-line treatment; and rK39 dipsticks are being distributed to peripheral health facilities to complement clinical diagnosis. With the revision of diagnosis and treatment guidelines, facilities are now able to obtain sodium stibogluconate by providing Pharmaciens sans Frontières with a positive rapid diagnostic test result, but they are encouraged to also take a blood sample for direct agglutination testing, as this is still considered more reliable (*8*). Meanwhile, the UK-based Malaria Consortium is providing long-lasting insecticide-treated nets to areas in Jonglei and Eastern Equatoria, where malaria and kala-azar are co-endemic.

Conclusions

A strong presence of international donors and the Southern Sudan government's desire to quickly reconstruct the healthcare sector provide ample opportunity to reduce the incidence of kala-azar. However, this goal can be achieved only with the necessary resources.

Acknowledgments

We thank all agencies and their staffs involved in the control of kala-azar in Southern Sudan, in particular, Médecins sans Frontières.

Dr Kolaczinski is the Neglected Tropical Diseases and Public Health Specialist of the Malaria Consortium, Africa. He has a broad interest in infectious disease epidemiology and the design, implementation, and evaluation of control programs in resource-poor settings.

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etymologia

Leishmaniasis

[lēsh-ma′-ne-ə-sis]

Disease caused by protozoan parasites of the genus *Leishmania*, named in 1901 for British Army doctor William Leishman, who developed a stain to detect the agent. It is transmitted by the bite of certain species of sand fly, including the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World.

Leishmaniasis has 2 major forms: cutaneous, characterized by skin sores, and visceral, which affects internal organs and is characterized by high fever, substantial weight loss, swelling of the spleen and liver, and anemia. If untreated, the disease is universally fatal within 2 years. Visceral leishmaniasis is also called kala-azar, a Hindi term meaning "black fever." The causal agent, *Leishmania donovani*, was also named for physician Charles Donovan, who discovered the agent in India in 1903. An estimated 500,000 cases occur each year; India has the greatest number, followed by Bangladesh, Brazil, Nepal, and Sudan.

Source: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; http://www.time.com/time/magazine/article/0,9171,987111-6,00.html; http://www.who.int/topics/leishmaniasis/en

Dengue Virus Type 4, Manaus, Brazil

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We report dengue virus type 4 (DENV-4) in Amazonas, Brazil. This virus was isolated from serum samples of 3 patients treated at a tropical medicine reference center in Manaus. All 3 cases were confirmed by serologic and molecular tests; 1 patient was co-infected with DENV-3 and DENV-4.

Dengue fever is the main arthropod-borne viral disease of humans and a resurgent global public health concern; an estimated 50–100 million cases occur every year, primarily in the tropical regions of the world (*1–3*). Dengue viruses (DENVs) belong to the genus *Flavivirus*, family *Flaviviridae*. They are single-stranded, positive-sense, RNA viruses grouped into 4 antigenically related, but distinct, serotypes (DENV-1 to DENV-4) (*1*).

DENV infection has increased in Brazil in the past decade, particularly after 1994, as a result of *Aedes aegypti* dissemination. Vector dispersion was followed by introduction of DENV-1, DENV-2, and DENV-3 in major Brazilian cities. Co-circulation of different serotypes has caused cases of the more severe forms of dengue, namely, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (*4*). Two dengue epidemics occurred in Manaus (3°5′S, 60°W), the capital of the state of Amazonas (Figure), during 1998–1999 and in 2001 (*5,6*). DHF cases were observed in association with DENV-1 and DENV-2 in the most recent epidemic. Currently, DENV-3 also co-circulates in Manaus (*7*).

The Study

A study on serologic and molecular characterization of DENV isolates was initiated in January 2005 at the Fundação de Medicina Tropical do Amazonas (FMTAM). Parasite-negative patients who had clinical symptoms of malaria were invited to participate in the study. Each patient received essential information and signed a consent form approved by the FMTAM ethical committee.

All serum samples were collected during the acute phase of illness and tested for DENV infection by using 3 methods. The first method was virus culture, for which serum samples were placed on the *Aedes albopictus* cell line C6/36 grown in Leibovitz-15 medium containing 5% fetal bovine serum, followed by viral antigen identification with type-specific monoclonal antibodies in an indirect immunofluorescence assay (*8*). The second method was detection of immunoglobulin M antibodies to DENV by an ELISA on serum samples from patients ≥7 days after onset of symptoms (*9*). The third method was nucleic acid amplification and typing by a seminested reverse transcription–PCR (RT-PCR) protocol on the basis of that described by Lanciotti et al. (*10*).

Briefly, viral RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), and reverse transcription was conducted on 5 μL of extracted RNA with Superscript III (Invitrogen, Carlsbad, CA, USA) and random primers. After incubation for 1 h at 50°C, 2 μL of each cDNA was subjected to PCR amplification with D1 and D2 primers for 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final extension for 10 min at 72°C. A second round of amplification was conducted with 10 μL (diluted 1:100) of the first amplicon, a mixture of type-specific reverse primers (TS1–TS4), and the conserved forward primer D1. The same cycling parameters were used as in the first reaction.

DENV-4 was detected in 3 samples (AM750, AM1041, and AM1619) by virus culture or RT-PCR. It was identified as a co-infecting virus with DENV-3 in isolate AM750; samples AM1041 and AM1619 represented single DENV-4 infections (Table). To confirm these results, samples were reamplified with each PCR typing primer separately. Generated amplicons were cloned into a TA vector (Invitrogen), and ≥3 colonies for each sample were sequenced in both directions by using the BigDye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA). DENV-3 and DENV-4 nucleotide sequences obtained were subjected to a basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/) analysis that used the megablast algorithm optimized for highly similar sequences. Using this approach, we obtained sequences with identities ranging from 95% to 99% for DENV-3 and 94% to 98% for DENV-4 for isolates AM750-D3, AM750-D4, and AM1619. These results confirmed our results obtained with monoclonal antibodies and PCR typing assays. The nucleotide sequences were deposited in GenBank under accession nos. EU127898 (AM750-D3), EU127899 (AM750-D4), and EU127900 (AM1619).

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Figure. Location of Amazonas State and Manaus City, Brazil. CO, Colombia; VE, Venezuela; RR, State of Roraima.

The 3 DENV-4–positive samples were obtained from patients who lived and worked in Manaus and reported no travel history for ≥15 days before onset of symptoms. These samples were obtained during another study that identified 62 DENV-positive samples from January 2005 through June 2007 (24 DENV-2, 35 DENV-3, and the 3 DENV-4 cases in our study) among 128 samples tested from patients in 14 municipalities in Amazonas.

Conclusions

Since its introduction into the Western Hemisphere in 1981, DENV-4 has been associated with dengue fever and only sporadically associated with serious cases of DHF or DSS (*1*). A study in Colombia found more DHF patients infected with DENV-2 than with DENV-3 or DENV-4 (*11*). Conversely, another study showed an association of DENV-4 with an epidemic of DHF that occurred in Mexico in 1984 (*12*).

There are many host (and perhaps viral) factors in dengue infections that may lead to development of DHF. On the basis of the antibody-dependent enhancement hypothesis, the most important factors would be those generated by the patient's immune response upon secondary infections (*13*). The 3 isolates reported in our study were from patients with no travel history, which indicates that DENV-4 is present in Manaus. Detection of DENV-4 in Brazil co-circulating with other DENV serotypes endemic to this country represents an increased risk for DHF or DSS because many persons have been sensitized by previous dengue infections but are not protected against infection with DENV-4.

The first report of DENV-4 in Brazil was in the state of Roraima in 1982. Since that time, no other isolate of DENV-4 has been reported in any part of Brazil (*14,15*). The resurgence of DENV-4 in the Amazon region of Brazil most likely resulted from the proximity of Brazil to DENV-4–endemic countries (Venezuela and Colombia). Additional genotyping studies are being conducted to verify this assumption and to obtain information on dengue epidemiology in Brazil.

Our study documents the detection of DENV-4 in Manaus, Amazonas, and the first isolation of this serotype in Brazil in 25 years. These findings reinforce the need for continual epidemiologic studies and use of classic and molecular approaches in the surveillance of emerging or re-emerging diseases.

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Table. Results of different methods used to confirm dengue virus type 4 (DENV-4) infection, Manaus, Amazonas, Brazil*

Isolate	IgM antibody capture ELISA	Virus culture†				M-PCR				S-PCR				BLAST
		D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4	
AM750	–	–	–	+	+	–	–	+	+	–	–	+	+	DENV-3/ DENV-4‡
AM1041	+	–	–	–	+	–	–	–	+	–	–	–	+	NS
AM1619	+	–	–	–	+	–	–	–	+	–	–	–	+	DENV-4

*IgM, immunoglobulin M; M-PCR, multiplex typing PCR described by Lanciotti et al. (*10*); S-PCR, single serotype-specific primer PCR; D, dengue serotype; BLAST, basic local alignment search tool; NS, not sequenced.
†Viral antigens detected by immunofluorescence with type-specific monoclonal antibodies.
‡Isolate AM750 is from a person with a co-infection; different clones were sequenced.

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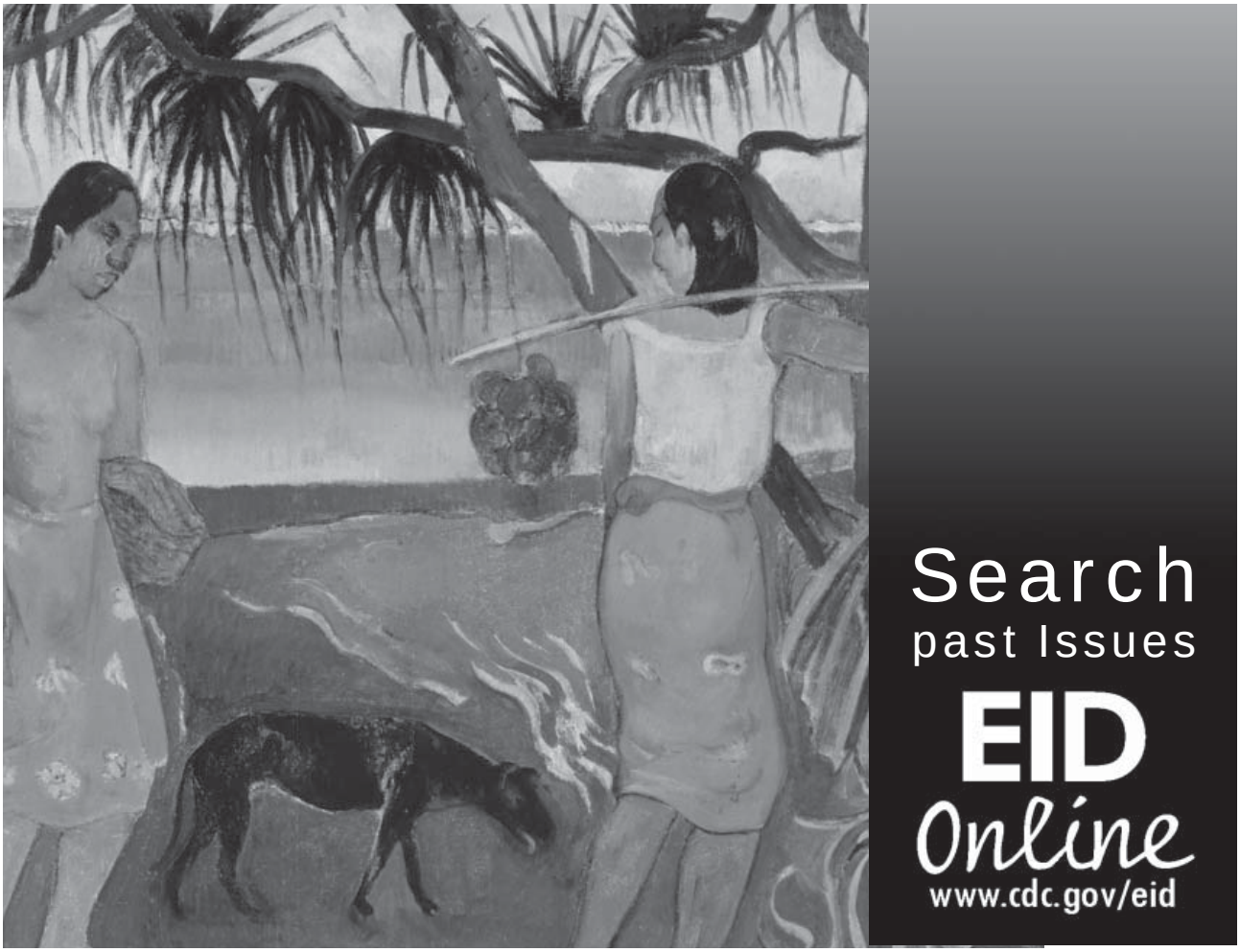

Rat-to-Elephant-to-Human Transmission of Cowpox Virus

To the Editor: Despite the eradication of smallpox in the past century, other orthopoxviruses, such as monkeypox virus, vaccinia virus in Brazil, and cowpox virus (CPXV) in Europe (*1*), still infect humans. CPXV has been restricted to the Old World with wild rodents as its natural reservoir (*2*,*3*). Human CPXV infections are commonly described in relation to contact with diseased domestic cats, rarely directly from rats (*2*,*4*). Human infections usually remain localized and self-limiting but can become fatal in immunosuppressed patients (*5*). CPXV infections in captive exotic animals have been reported to be transmitted by rodents (*2*,*6*).

In February 2007, a circus elephant (*Elephas maximus*) in northern Germany exhibited disseminated ulcerative lesions of the skin and mucosal membranes (Figure, panel A) caused by CPXV infection; the elephant was euthanized after treatment attempts failed. Electron micrographs of negative-stained biopsy specimens of tongue lesions showed orthopoxvirus particles. The presence of orthopoxvirus after routine virus isolation in Hep2 cells was confirmed in direct immunofluorescence assay with orthopox-specific antibodies. The morphologic feature of hemorrhagic pocks on the chorioallantoic membrane (CAM) of infected embryonated hen's eggs indicated CPXV. This finding was confirmed by sequence analysis of the complete hemagglutinin (HA) open reading frame (ORF), which showed 99% homology of 921 bp to CPXV isolated in 1984 from an elephant in Hamburg, Germany (Figure, panel B). A serum sample was drawn from the elephant 2 weeks before euthanasia. An indirect fluorescent antibody test (IFAT) detected immunoglobulin (Ig) G antibodies against the new corresponding elephant virus isolate (termed CPXV GuWi) with a titer of 1,260. According to the owner, the >40-year-old female elephant had never been vaccinated with vaccinia virus.

Eight days after the elephant's death, a circumscribed lesion developed on the back of the right hand of a 19-year-old immunocompetent, healthy, unvaccinated animal keeper. CPXV was isolated from lesion fluid and was found to be homologous by using the HA ORF to CPXV GuWi. A convalescent-phase serum sample from the keeper taken 3 weeks later showed a significant increase in IgM

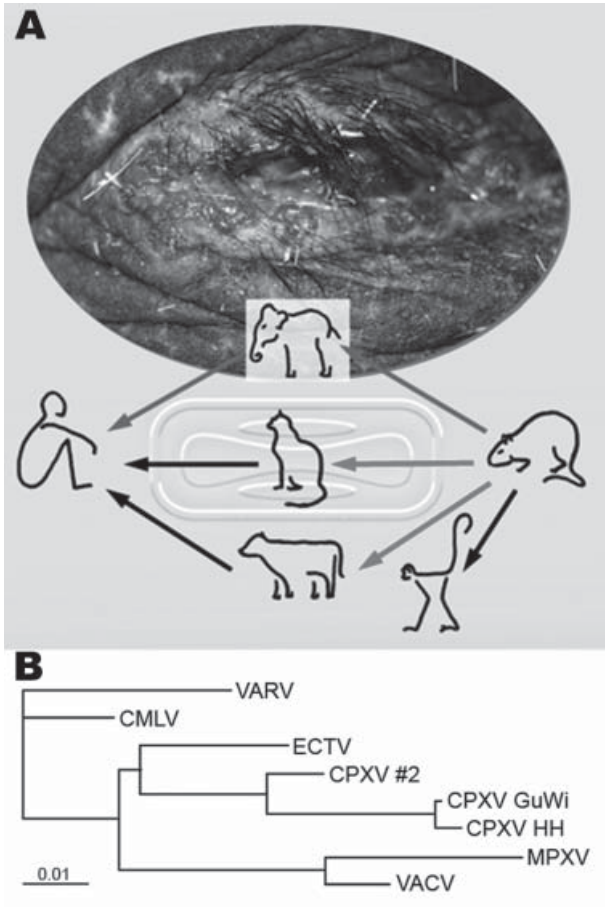

Figure. Route of cowpox virus (CPXV) transmission and phylogenetic analysis of orthopoxviruses. A) Disseminated ulcerative lesions of the skin around the eye of the circus elephant. Although transmission of CPXV has been confirmed from cats and cows to humans (*1*,*2*), transmission from rodents, commonly mice, to cats and cows is suspected but still unproven (*3*). Rats have been confirmed as vectors for CPXV transmission to monkeys and humans (*4*,*7*). A complete chain of CPXV infection is verified from rat to elephant and from elephant to human. B) Phylogenetic tree of nucleotide sequences of the complete hemagglutinin open reading frame (921 bp) from CPXV isolates from the elephant and rat (CPXV GuWi), and additional poxviruses available in GenBank: VARV (variola major virus, strain Bangladesh-1975; L22579), CMLV (camelpox virus M-96, Kazakhstan; AF438165.1), ECTV (ectromelia virus, strain Moscow; AF012825.2), CPXV HH (cowpox virus cowHA68, Hamburg; AY902298.2), MPXV (monkeypox virus, strain Zaire-96-I-16; AF380138.1), and VACV (vaccinia virus WR; AY243312). In addition, the complete sequence of the hemagglutinin gene obtained from a different human CPXV case (CPXV #2) found in that area is shown. Nucleotide sequences were aligned and analyzed by using the BioEdit software package (www.mbio.ncsu.edu/BioEdit/bioedit.htm). A multiple alignment was analyzed with the neighbor-joining method. The branch length is proportional to evolutionary distance (scale bar).

(from 40 to 2,560), IgG (from 20 to 10,240), and neutralizing antibody (from <5 to 80) titers.

Further simultaneous investigations were undertaken to determine the source of infection. Because no felids were kept on the circus premises, the focus centered on wild rodents that had propagated and infested the area because of the mild winter. Six days after the elephant's death, 4 rats (*Rattus norvegicus*) were caught and tested for orthopoxvirus antibodies. Although none of the rats had epidermal lesions or other pathologic changes indicative of a poxvirus infection, all were tested by IFAT and found to be serologically positive (IgG titers 40, 320, 2,560, and >10,240; IgM titers <5, <5, 160, and 2,560), indicating a recent infection in at least 2 animals. CPXV-typical pock morphologic features on the CAM could be visualized after homogenized liver and spleen of the animal with the highest titer was passaged 3 times. Infected CAM and original organ tissues (liver and spleen) showed CPXV by PCR and subsequent sequencing. The corresponding HA ORF displayed perfect homology to the viruses isolated from the elephant and the animal keeper.

We report CPXV infection in humans transmitted from an elephant, with rats as a probable source of the elephant's infection (Figure, panel A). Although the animal keeper was infected by direct contact with the elephant, the exact transmission route from rat to elephant remains unclear. Nevertheless, rats have proven to be a natural reservoir for CPXV (*4*,*7*), and infections persisting for >3 weeks were shown for other rodents (*8*). No data about CPXV prevalence in rats are available, and no data for CPXV isolates from rats have been published in Germany. Therefore, further studies on rats as CPXV reservoir are needed to estimate the potential risk for infection among humans and exotic animals. Zoo and circus animals, especially elephants, seem to be highly susceptible to generalized CPXV infections. Although modified vaccinia virus Ankara was authorized in Germany to be used in vaccinating exotic animals (*9*), this case highlights the need for increased efforts toward general vaccination of potentially susceptible exotic animals in Europe.

The sequence identity of the HA ORFs also demonstrates a low mutation rate of CPXV after it crosses species barriers. As the Figure, panel B, infers, there is a phylogenetic difference between CPCV GuWi and CPXV from a human patient living in the same geographic area (CPXV #2), which indicates the cocirculation of >1 CPXV variant (*9*,*10*). Considering the extremely high virus load in infected animals, the broad host range of CPXV, and the abandoned vaccination against smallpox, this case emphasizes the risk among humans of acquiring CPXV infection (*6*). It also highlights the need for increased awareness regarding clinical features of orthopoxvirus infections and the importance of developing new antiviral drugs against orthopoxviruses.

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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 one Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Avian Influenza Knowledge among Medical Students, Iran

To the Editor: Avian influenza is an infectious disease caused by type A strains of influenza virus (*1*). Since January 2004, Thailand and several other Southeast Asian countries have experienced outbreaks of avian influenza in poultry, and >100 million poultry have been culled or have died (www.who.int/csr/disease/avian_influenza/en). The prevalence of severe and fatal cases involving bird-to-human transmission is increasing (*2*). Experts fear that the avian influenza virus now circulating in Asia will mutate into a highly infectious strain and pass not only from animals to humans, but also among humans, which would lead to a pandemic (*3*).

During a pandemic, public health agencies and medical students will play critical roles in controlling the spread of disease (*4*). Therefore, medical school curricula should include specific courses in the epidemiology of avian influenza to ensure that all medical students and health care professionals will have the knowledge needed to confront a potential pandemic. In Iran, medical education comprises basic sciences (first to third year), externship (fourth to fifth year, preclinical education), and internship (sixth to seventh year). Medical students study virology during the second year of medical school. Thereafter, no additional coursework in virology is offered. Because several cases of avian influenza have been found in adjacent countries such as Turkey and Iraq, we anticipate that the virus will spread to Iran. Therefore, we designed a study to assess the knowledge of a group of Iranian medical students regarding avian influenza and to delineate the potential source of their knowledge.

The study population comprised second- and third-year medical students at the Faculty of Medicine, Tabriz University of Medical Sciences, in May 2006. We used a self-administered questionnaire that was based on information obtained from a review of the literature on avian influenza. This questionnaire (Table) comprised 3 sections: 1) demographic information, including age and sex of participants (2 items); 2) avian influenza–related questions covering general information, history, modes of transmission, clinical symptoms, and prevention (18 items); and 3) a multiple-choice question regarding the students' source of information about avian influenza (1 item). (As shown, the questionnaire used the common parlance "bird flu" for avian influenza.) Possible responses for section 2 included "yes," "no," and "I don't know." The knowledge score was calculated by giving +1 for a correct answer, –1 for an incorrect answer, and zero for "I don't know" responses. A total of 18 points could be achieved if all questions in section 2 were correctly answered. Higher scores indicated a greater level of knowledge. We invited 2 epidemiologists and 1 statistician to qualify and examine the questions. Data were pre-

Table. Respondents' knowledge of avian influenza (n = 234), Iran, May 2006

Questions	Correct answer	% Yes	% No	% Don't know
History				
1. The first case of human infection with bird flu virus occurred in Hong Kong in 1997.	Yes	27.7	3.2	69.1
2. Most fatal cases of bird flu have been reported in Vietnam.	Yes	28	2.2	69.9
General information				
3. Influenza virus occurs naturally among wild birds.	Yes	14.3	63.7	22
4. Bird flu may be transmitted into other mammals such as horses and pigs.	Yes	25.3	19.8	54.9
Transmission				
5. Transmission of the disease from person to person is possible.	Yes	47.3	19.8	33
6. Main modes of transmission are through saliva and nasal secretions.	Yes	54.2	13.5	32.3
7. Bird flu virus can be transmitted into persons through the alimentary tract.	No	74.2	15.7	10.1
8. Bird flu is transmitted into humans through handling and cleaning of contaminated game.	Yes	41.3	37	21.7
9. The consumption of contaminated chicken as broiler would have the risk of affliction.	Yes	72.2	21.1	6.7
10. Cooking eggs as soft-boiled eliminates the virus.	No	19.1	68.5	12.4
Diagnosis				
11. A laboratory test is needed to confirm bird flu in humans.	Yes	10	83	7
Clinical presentations				
12. Respiratory tract is the main infected system in the body.	Yes	59.8	9.2	31
13. The incubation period of bird flu is ≈7 days.	Yes	13.6	2.3	84.1
14. Symptoms of bird flu in humans are similar to seasonal influenza.	Yes	20	11	69
15. Bleeding from the nose and bleeding from the gums are early symptoms of bird flu.	Yes	2	13	85
16. Bloody diarrhea (dysentery) is one of the manifestations of bird flu.	No	30.8	8.8	60.4
Prevention				
17. Bird flu is a preventable infection.	Yes	86	4.3	9.7
18. There is a vaccine to protect humans from bird flu virus.	No	20.9	37.4	41.8

sented in mean ± standard deviation or percentage when appropriate. Statistical analysis was performed by SPSS Windows version 12.0 (SPSS Inc., Chicago, IL, USA) using the χ^2 test; p value was set at 0.05.

Two hundred thirty-four of 252 second- and third-year medical students completed the questionnaire. The mean age of the respondents was 19 ± 0.87 years (range 18–23). Twenty-nine percent (n = 68) of the students were male and 71% (n = 166) were female.

The mean knowledge score was 4.76 of 18 (total of correct and incorrect responses) (range –6 to 11). Second- and third-year students comparably responded to 16/18 questions (χ^2 test). A list of questions and the percentage of students' responses are provided in the Table.

Most of the respondents (67.2%) indicated that mass media (radio, television, and newspapers) was their major source of information about avian influenza, followed by scientific books and journals (8.3%), the Internet, (13%), and family and friends (10.4%). Only 1.1% of the medical students mentioned "school educational materials" as the source of their information.

Our study shows a relatively low level of knowledge of avian influenza among a group of Iranian medical students. Surprisingly, mass media was the main source of information in our study. Training health care professionals as well as medical students is of great importance in controlling infectious diseases. The findings of this study should be considered seriously by local health centers and disease control agencies because preparing health care professionals with sufficient knowledge is essential to confronting a potential pandemic. We believe that the low level of knowledge about avian influenza among these medical students is primarily a reflection of insufficient academic courses in the medical school curriculum. We strongly recommend improving the quality of education on this topic through access to textbooks, articles, seminars, and specific courses.

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Lorraine Strain of *Legionella pneumophila* Serogroup 1, France

To the Editor: Legionellosis is a pneumonia caused by inhalation of *Legionella* spp. in aerosol water particles. *Legionella pneumophila* is responsible for ≈90% of cases; serogroup 1 alone accounts for ≈85% of cases (*1*). Epidemiologic analyses based on pulsed-field gel electrophoresis (PFGE) and sequence-based typing of clinical isolates of *L. pneumophila* serogroup 1 have detected sporadic, epidemic, and endemic strains (*2*). Most cases are sporadic and are associated with strains that have not been identified. A strain is considered endemic to an area when several isolates that have identical PFGE patterns and that cause several epidemiologically unrelated cases of legionellosis are detected in that area. Since 1998, the most prevalent strain endemic to France has been the Paris strain (*3*), which was responsible for 12.2% of culture-confirmed cases of legionellosis from 1998 through 2002 (*3*). The Paris strain has also been detected in clinical samples from several other European countries (Switzerland, Italy, Spain, and Sweden) and in environmental samples (*3*,*4*).

We identified a new endemic clone of *L. pneumophila* serogroup 1, the Lorraine strain, and report its spread throughout France. The French national reference center for *Legionella* collects all clinical isolates of *Legionella* spp. as part of an epidemiologic surveillance system. All *L. pneumophila* serogroup 1 isolates are typed by PFGE methods as described (*4*). When necessary, sequence-based typing (*5*,*6*) and monoclonal antibody–based (MAb) subgrouping are also used (*7*).

From 1995 through 2006, the reference center typed 1,768 clinical *Legionella* isolates by means of PFGE. Most PFGE patterns were unique and thus corresponded to sporadic cases. Another 145 (8.2%) patterns were identical and corresponded to the endemic Paris strain. An identical PGFE pattern was also found for 80 (4.5%) isolates from epidemiologically unrelated patients; these isolates were further characterized by sequence-based typing and MAb subgrouping. Sequence type was deduced for the following genes: *flaA, pilE, asd, mip, mompS, proA,* and *neuA* (*6*). The sequence type was obtained for 78 of the 80 isolates and was 5, 10, 22, 15, 6, 2, 6. The sequence type of the remaining 2 isolates differed from that of the other 78 by 2 alleles (*pilE* and *proA*) and was 5, **1**, 22, 15, 6, **10**, 6

(**boldface** indicates differences). All but another 2 isolates (which belonged to the Benidorm subgroup) belonged to the Allentown MAb subgroup. Hence, the new endemic strain, Lorraine, was represented by 76 isolates that had an identical PFGE pattern, sequence type, and MAb subgroup.

Isolation of the Lorraine strain was reported anecdotally before 2002. Since 2002, the prevalence of this strain in France has increased considerably, accounting for 10.5% clinical isolates in 2005 and 9.0% in 2006 (Figure). In contrast, prevalence of the Paris strain was ≈10% from 1998 through 2002 and peaked in 2000 (16.9%) in association with a hospital outbreak in Paris. From 2003 through 2006, prevalence of the Paris strain fell to ≈6.5%.

The Lorraine strain has caused 2 outbreaks. In the first, 3 isolates were recovered from respiratory samples of 34 patients for whom legionellosis was diagnosed by urinary antigen testing in Lyon in 2005. The second outbreak occurred in a western suburb of Paris in 2006, when 1 isolate was cultured from respiratory samples of 12 patients whose diagnoses were also made by urinary antigen testing.

From 1995 through 2006, >4,000 environmental *Legionella* isolates in France were typed by PFGE, and >700 types were identified. The Paris strain type was identified >500 times, but the Lorraine type was identified in only 3 water samples, including 1 from the cooling tower responsible for the outbreak in the Paris suburb. The Lorraine strain is thus rarely found in water samples, which hinders environmental investigations of its sources in outbreaks of legionellosis.

A similar disparity between the clinical and environmental distribution of *Legionella* strains has been reported (*8*). In a collection of 284 unrelated clinical isolates and 117 unrelated environmental isolates, Harrison et al. found that 3 types, identified by restriction fragment length polymorphism, accounted for 40% of clinical isolates but only 18% of environmental isolates (*8*).

The high prevalence of the Lorraine strain in clinical samples and its extremely rare detection in water samples have several possible explanations: 1) this strain could be related to specific host factors; 2) it could be highly virulent even in low amounts, below the culture detection limit; and 3) it could be more susceptible than other strains to different stressors (e.g., biocide treatment, selective preplating techniques, environmental medium specific components).

In conclusion, prevalence of a new *L. pneumophila* serogroup 1 strain, Lorraine, endemic to France, is increasing in clinical samples although rarely detected in water samples. The type strain, Lorraine (CIP108 729), is available from the strain collection of the Pasteur Institute (Paris, France).

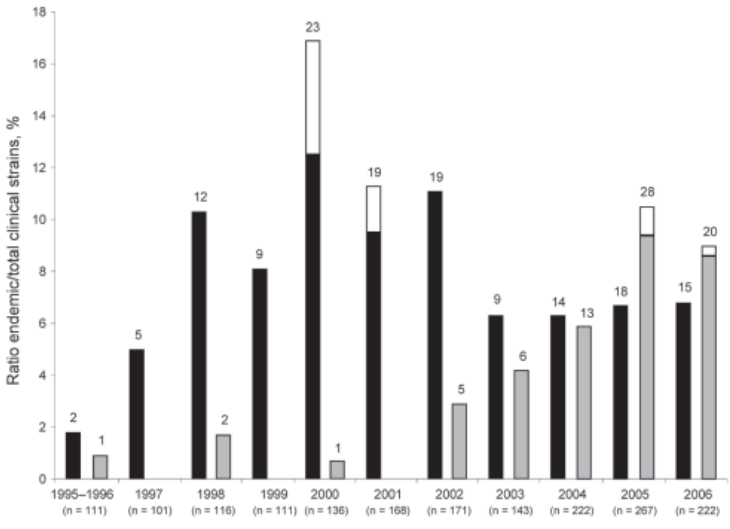

Figure. Prevalence of the *Legionella pneumophila* Paris (black bars) and Lorraine (grey bars) endemic strains, France, 1995–2006. White bar sections represent the proportion of strains isolated during outbreaks. For example, in 2000 the Paris strain accounted for 16.9% of clinical isolates: 12.5% unrelated and 4.4% related to the same outbreak. Numbers above each bar indicate the number of isolates.

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Bluetongue in Captive Yaks

To the Editor: In August 2006, several northern European countries including Belgium reported cases of bluetongue (BT) (*1*). This noncontagious, arthropod-borne animal disease is caused by *Bluetongue virus* (BTV), genus *Orbivirus,* family *Reoviridae*. The genome of BTV consists of 10 segments of double-stranded RNA; 24 serotypes have been reported (*2*). Serotype 8 (BTV-8) was implicated in the emergence in Belgium (*3*). All ruminant species are thought to be susceptible to BT (*2*). We report laboratory-confirmed clinical cases of BT in yaks (*Bos grunniens grunniens*).

Yaks living in captivity in a Belgian animal park showed clinical signs of BT. A clinical examination performed on 1 yak showed loss of weight associated with a progressive weakness linked to anorexia, ulcerative and necrotic lesions on the muzzle with some crusts and mucopurulent nasal discharge, and udder erythema with papules and crusts. The tongue was severely swollen and cyanotic and protruded from the mouth (Figure). The animal was reluctant to move and was recumbent (possibly as a consequence of podal lesions linked to BT); it died 7 days after examination. Necropsies were performed on carcasses of this and another yak. The main lesions found were severe diffuse congestion of the lungs with edema and emphysema, acute hemorrhagic enteritis restricted to the ileum and jejunum, and petechial hemorrhages on the abomasums. No lesions characteristic of coronitis were noted.

Samples of spleen and bone marrow were taken and prepared according to the method of Parsonson and McColl (*4*). A real-time reverse transcription quantitative–PCR (RT-qPCR) targeting BTV segment 5 (RT-qPCR_S5) was used to detect BTV RNA in tissues samples. Each test was performed in parallel with a RT-qPCR that amplifies β-actin mRNA as an internal control (RT-qPCR_ACT). Both assays were conducted according to Toussaint and others (*5*), with slight modifications. Briefly, total RNA was purified from 25 mg of tissue by Trizol extraction (Invitrogen, Carlsbad, CA, USA) and denatured by heating for 3 min at 95°C with 10% dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription reactions were conducted by using the Taqman reverse transcription reagents according to the manufacturer instructions (Applied Biosystems, Foster City, CA, USA). RT-qPCR reactions consisted of 1× concentrated Taqman fast universal PCR master mix (Applied Biosystems), 375 nM (β actin) or 500 nM (BTV) of each primer, 250 nM Taqman probe, and 5 μL cDNA. Cycling conditions were as follows: 1 cycle at 95°C for 20 s, followed by 45 cycles of 1 s at 95°C, and 20 s at 60°C. The specificity of the RT-qPCR used had been previously tested against prototype strains of genetically related viruses (9 strains of epizootic hemorrhagic disease virus

Figure. A captive yak infected with bluetongue virus. Tongue is swollen, cyanotic, and protruding from the mouth.

and 9 strains of African horse sickness virus) (*5*). The RT-qPCR tests confirmed BTV viremia.

The yak species in its natural biotope is usually rarely exposed to competent *Culicoides* vectors. Antibodies against BTV have been found in many wild ruminants (*6*), and our results extend the number of ruminant species susceptible to BTV. In the northern European BT outbreak, lesions in cattle and sheep were mainly localized to the regions of the muzzle, mouth, and eye; clinical signs were not always obvious (*7*,*8*). As in cattle and sheep, clinical signs in yaks were observed on the muzzle, in the periocular region, and around and inside the mouth. These signs clearly reflected viral-induced endothelial damage triggering disseminated intravascular coagulation and a hemorrhagic diathesis commonly described in sheep and cattle (*2*). In our case, lesions depicted pronounced microvascular damage. According to the severity of the lesions and rates of illness and death observed, the yak, like sheep, appears to be highly susceptible to BTV.

In the epidemiology of BT in African countries, cattle and wild ruminant species such as antelopes play a role as asymptomatic reservoir hosts of the virus (*2*). Some wild ruminant species in captivity could also play this role in European countries affected by the recent BT outbreak. These cases could be of particular concern for all parks and zoos that gather numerous wild ruminants. Illness, reproductive failure, and deaths usually reported with BT (*9*) could generate substantial losses on these premises. Moreover, the source of BTV-8 in the northern European outbreak remains unclear, and the role of wild ruminant species has to be taken into account. In the future, European authorities should consider vaccination to prevent the spread of the disease in European member states (*10*). All premises with wild ruminants need to be involved in BT control and prophylaxis.

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Murine Typhus, Algeria

To the Editor: Rickettsioses, or typhoid diseases, are caused by obligate intracellular bacteria of the order Rickettsiales. The ubiquitous murine typhus is caused by *Rickettsia typhi*. Although cat fleas and opposums have been suggested as vectors in some places in the United States, the main vector of murine typhus is the rat flea (*Xenopspylla cheopis),* which maintains *R. typhi* in rodent reservoirs (*1*). Most persons become infected when flea feces containing *R. typhi* contaminate broken skin or are inhaled, although infections may also result from flea bites (*1*). Murine typhus is often unrecognized in Africa; however, from northern Africa, 7 cases in Tunisia were documented in 2005 (*2*).

We conducted a prospective study in Algeria which included all patients who had clinical signs leading to suspicion of rickettsioses (high fever, skin rash, headache, myalgia, arthralgia, eschar, or reported contact with ticks, fleas, or lice) who visited the Oran Teaching Hospital in 2004–2005 for an infectious diseases consultation. Clinical and epidemiologic data as well as acute-phase (day of admission) and convalescent-phase (2–4

weeks later) serum samples were collected. Serum samples were sent to the World Health Organization Collaborative Center for Rickettsial Diseases in Marseille, France. They were tested by immunofluoresence assay (IFA), by using spotted fever group (SFG) rickettsial antigens (*R. conorii conorii*, *R. conorii israelensis*, *R. sibirica mongolitimonae*, *R. aeschlimmanii*, *R. massiliae*, *R. helvetica*, *R. slovaca*, and *R. felis*) and *R. typhi* and *R. prowazekii* as previously reported (*3*). When cross-reactions were noted between several rickettsial antigens, Western blot (WB) assays and cross-absorption studies were performed as previously described (*4*). A total of 277 patients were included. We report 2 confirmed cases of *R. typhi* infection in patients from Algeria.

The first patient, a 42-year-old male pharmacist who reported contact with cats and dogs parasitized by ticks, consulted with our clinic for a 10-day history of high fever, sweating, headache, arthralgia, myalgia, cough, and a 6-kg weight loss. He had not received any antimicrobial drugs before admission. No rash, eschar, or specific signs were found. Standard laboratory findings were within normal limits. No acute-phase serum sample was sent for testing. However, IFAs on convalescent-phase serum were negative for SFG antigens (except *R. felis*: immunoglobulin [Ig] G 64, IgM 128), but they showed raised antibodies against *R. typhi* and *R. prowazekii* (IgG 2,048, IgM 1,024).

The second patient, a 25-year-old farmer, was hospitalized for a 5-day history of fever, headache, diarrhea, and lack of response to treatment with amoxicillin and acetaminophen. He reported contact with cats and cattle. A discrete macular rash and pharyngitis were observed. Standard laboratory findings were within normal limits, except neutrophil count was elevated at 11.2/μL (normal levels 3–7/μL). Acute-phase serum was negative for rickettsial antigens. Convalescent-phase serum obtained 2 weeks later was positive for several SFG antigens (IgM only; the highest level was 256 for *R. conorii*), and higher levels of antibodies were obtained against *R. typhi* and *R. prowazekii* (IgG 256, IgM 256). WB and cross-absorption studies confirmed *R. typhi* infection (Figure). Both patients recovered after a 3-day oral doxycycline regimen and have remained well. (A single 200-mg dose of oral doxycycline usually leads to defervescence within 48–72 hours [*1*]).

Murine typhus is a mild disease with nonspecific signs. Less than half of patients report exposure to fleas or flea hosts. Diagnosis may be missed because the rash, the hallmark for rickettsial diseases, is present in <50% of patients and is often transient or difficult to observe. Arthralgia, myalgia, or respiratory and gastrointestinal symptoms, as reported here, are frequent; neurologic signs may also occur (*5*). As a consequence, the clinical picture can mimic other diseases. A review has reported 22 different diagnoses that were proposed for 80 patients with murine typhus in the United States (*6*).

Serologic tests are the most frequently used and widely available methods for diagnosis of rickettsioses (*7*). IFA is the reference method (*7*). However, *R. typhi* may cross-react with other rickettsial antigens, including SFG rickettsiae, but especially with the other typhus group rickettsia, *R. prowazekii*, the agent of epidemic typhus (*8*). Epidemic typhus is transmitted by body lice and occurs more frequently in cool areas, where clothes are infrequently changed, and particularly during human conflicts. It is still prevalent in Algeria (*9*).

This cross-reactivity led to some difficulties in interpreting serologic results (*10*). However, WB and cross-adsorption studies can be used when cross-reactions occur between rickettsial antigens. They are useful for identifying the infecting rickettsia to the

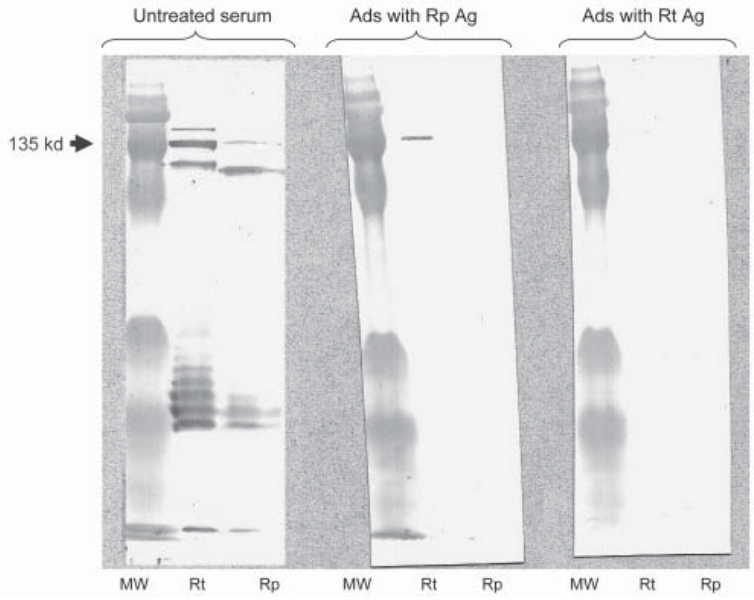

Figure. Western blot assay and cross-adsorption studies of an immunofluoresence assay–positive serum sample from a patient with rickettsiosis in Algeria. Antibodies were detected at the highest titer (immunoglobulin [Ig] G 256, IgM 256) for both *Rickettsia typhi* and *R. prowazekii* antigens. Columns Rp and Rt, Western blots using *R. prowazekii* and *R. typhi* antigens, respectively. MW, molecular weight, indicated on the left. When adsorption is performed with *R. typhi* antigens (column Ads with Rt Ag), it results in the disappearance of the signal from homologous and heterologous antibodies, but when it is performed with *R. prowazekii* antigens (column Ads with Rp Ag), only homologous antibody signals disappear, indicating that the antibodies are specific for *R. typhi*.

species level and for providing new data about the emergence or reemergence of rickettsioses, as reported here. These assays are, however, time-consuming and only available in specialized reference laboratories.

Clinicians need to be aware of the presence murine typhus in Algeria, especially among patients with unspecific signs and fever of unknown origin. Tetracyclines remain the treatment of choice.

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Natural Co-infection with 2 Parvovirus Variants in Dog

To the Editor: Canine parvovirus (CPV) emerged in the late 1970s, presumably by mutations in feline panleukopenia virus, and became a major viral pathogen of dogs worldwide (*1*). Between 1979 and 1981, the original type 2 virus (CPV-2) was replaced by a new genetic and antigenic variant, type 2a (CPV-2a). Between 1983 and 1984, CPV-2a was replaced by type 2b (CPV-2b), which differs from type 2a by only 1 epitope located at residue 426 of the VP2 capsid protein (*2*). CPV-2 does not replicate in cats, but the new variants replicate in dogs and cats (*3*). Recently, an antigenic change has been observed in a new strain, CPV-2c, isolated from domestic dogs in Italy (*4*). This variant was also detected in Vietnam (*5*), other countries in Europe (*6*), and the United States (*7*). CPV-2c was recently detected in cats (*8*) and is characterized by a replacement of aspartic acid with glutamic acid at residue 426 of the VP2 capsid protein.

To identify CPV types 2, 2a, and 2b, PCR methods were developed (*9*). However, these methods could not distinguish type 2c from type 2b (*4*). Consequently, we used a PCR–restriction fragment-length polymorphism (RFLP) assay with endonuclease *Mbo*II. This enzyme can distinguish type 2c from other CPVs (*4*). Recently, a real-time PCR assay based on minor groove binder (MGB) probe technology was developed for rapid identification and characterization of the antigenic variants. This assay is based on 1 nucleotide polymorphism in the VP2 gene (*10*).

In June 2006, a 10-week-old female dog (PT-32/07) was brought to the veterinary clinic in Figueira da Foz, Portugal, with clinical signs of parvovirus infection, after an episode of gastrointestinal disease in her litter. Three littermates, also brought to the clinic, showed no signs of infection. None of the dogs were vaccinated against CPV. Clinical signs in dog PT-32/07 were lethargy, anorexia, vomiting, diarrhea, and a temperature of 39.3°C. Identical signs were observed in 1 littermate 3 days later; the 2 other dogs did not show any signs other than lethargy and loose stools.

Rectal swab samples from all dogs were screened for CPV by using an immunomigration rapid test (Synbiotics Corporation, Lyon, France). Two of the dogs showed negative results, and 2 showed positive results. Feces, serum, and lingual swab samples were positive for parvovirus DNA. DNA was quantified by using a real-time PCR with TaqMan technology performed in an i-Cycler iQ (Bio-Rad Laboratories, Milan, Italy).

CPV variants were characterized by using MGB probe technology. This technology uses type-specific probes labeled with different fluorophores (FAM and VIC) that can detect single nucleotide polymorphisms between CPV types 2a/2b and 2b/2c (*10*). MGB probes specific for type 2b were labeled with FAM in both type 2a/2b

and 2b/2c assays, and MGB probes specific for type 2a (type 2a/2b assay) and type 2c (type 2b/2c assay) were labeled with VIC.

All specimens from 1 dog (PT-32/07) were positive for the 2 variants of CPV type 2 (CPV 2b and CPV 2c). Conversely, of the 3 littermates, 2 were positive for CPV type 2b and 1 was positive for CPV type 2c in all samples (Table).

A conventional PCR and RFLP analyses were performed by using the method of Buonavoglia et al. (*4*) with known positive CPV-2b and CPV-2c samples as controls to confirm our findings. The 583-bp PCR product obtained from the coinfected dog by using primer pair 555for/555rev was digested with *Mbo*II. Digestion generated 2 fragments (≈500 and 80 bp) in all dog samples. The CPV-2c control sample showed 2 fragments (≈500 and 80 bp), and CPV-2b control sample was not digested with *Mbo*II.

We report CPV-2b and CPV-2c variants in samples from a dog with littermates that were positive for CPV-2b or CPV-2c during an episode of gastrointestinal disease. Co-infection with multiple CPV variants that showed high genetic diversity in the VP2 gene has recently been reported in a domestic cat (*8*). Continuous and rapid evolution of CPV may cause serious problems in diagnostic testing and vaccine efficacy. Antigenic variation may negatively affect vaccine efficacy if changes occur at major antigenic sites. Thus, continuous monitoring for novel genetic and antigenic virus types is needed. Additional studies are in progress to characterize nucleotide sequences of all CPV isolates from this case.

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Table. Detection by minor groove binder probe assay of CPV antigenic variants in different specimens from dogs from the same litter (10 weeks old), Portugal, 2006*

Dog	Vaccines	Rapid test result for CPV	Days in clinic	Clinical course	Samples	TaqMan probe FAM a/b	TaqMan probe FAM b/c	TaqMan probe VIC a/b	TaqMan probe VIC b/c	CPV
PT-15/07	None	–	7	Recovered	Feces	+	+	–	–	2b
					Lingual swab	+	+	–	–	2b
					Serum	+	+	–	–	2b
PT-16/07	None	+	7	Recovered	Feces	+	+	–	–	2b
					Lingual swab	+	+	–	–	2b
					Serum	+	+	–	–	2b
PT-17/07	None	+	7	Recovered	Feces	–	–	–	+	2c
					Lingual swab	–	–	–	+	2c
					Serum	–	–	–	+	2c
PT-32/07	None	–	7	Recovered	Feces	+	+	–	+	2b/2c
					Lingual swab	+	+	–	+	2b/2c
					Serum	+	+	–	+	2b/2c

*CPV, canine parvovirus.

WU Polyomavirus Infection in Children, Germany

To the Editor: The human polyomaviruses JC and BK are known to cause persisting infections, which are usually asymptomatic in immunocompetent patients but may lead to severe disease in those who are immunosuppressed (*1*). Recently, 2 novel viruses of the family *Polyomaviridae* were detected in respiratory samples and named KI (*2*) and WU polyomavirus (WUPyV) (*3*). To investigate the frequency of WUPyV infections in Germany, we examined nasopharyngeal samples from hospitalized children with acute respiratory diseases for WUPyV DNA.

The samples tested for WUPyV infection consisted of stored nasopharyngeal aspirates (NPA) of hospitalized children at the Children's Hospital, University of Würzburg. The samples had been received for routine screening of respiratory viruses from January 2002 through September 2005 and from January 2007 through July 2007. All samples were routinely tested for antigens of adenoviruses, influenza viruses A (fluA) and B, parainfluenza viruses 1–3, and respiratory syncytial virus (RSV) by indirect immunofluorescence assays (Chemicon, Temecula, CA, USA). Remaining NPA material was stored at –20°C. DNA was extracted from the samples by using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) and stored at –70°C for further testing. All samples were also tested for human bocavirus (hBoV) DNA by PCR (*4*).

WUPyV PCR was performed by using the primer pair AG0048 and AG0049 described by Gaynor et al. (*3*). PCRs were conducted in a 50-μL volume consisting of 5-μL extracted DNA, 1× Qiagen HotStar buffer (QIAGEN, Hilden, Germany), dNTPs at final concentrations of 200 μmol/L each, 200 pmol of each primer, and 1.5 U of HotStarTaq polymerase. The cycling conditions were 50 cycles (94°C for 30 s, 53°C for 40 s, and 72°C for 1 min) after a preheating step of 10 min at 95°C. All PCR products of positive reactions by agarose gel electrophoresis with ethidium bromide staining were sequenced completely in both directions for confirmation of sequence specificity. One negative control was extracted and amplified for every 5 NPA samples. A plasmid containing the cloned PCR product was used as positive control. The sensitivity of the WUPyV PCR was 8.8 copies per reaction as determined by probit analysis, which corresponds to 440 copies per mL of sample. The study was approved by the ethics committee of the medical faculty at the University of Würzburg.

During the study period, 1,326 NPA of hospitalized children with febrile respiratory tract diseases were received for viral diagnostic evaluation. The median age of the patients was 1.6 years (mean age 3.2 years; range 7 days–22 years), and 58.4% were boys. DNA of 1,277 NPA from 1,085 children was available for retrospective testing. Of these, 62 (4.9%) samples from 59 children were positive by WUPyV PCR and subsequent sequencing. The median age of the WUPyV-positive children was 3.0 years (mean 2.9 years; range 4 months–6.3 years) (Figure), and 57% were boys. Of the children with WUPyV-positive NPA, 3.2% were >6 years of age, although children in this age group constituted 15.7% of the total population. Infections with WUPyV were found year round, but most occurred in the winter months. Yearly frequencies (July–June) of WUPyV-positive results varied from 3.2% to 8.5% during the observation period. These variations were not statistically significant. In 34 (54.8%) of the WUPyV-positive samples, co-infections with other respiratory viruses were detected, most frequently with adenovirus (n = 10) and fluA (n = 10), followed by hBoV (n = 9) and RSV (n = 5). The co-infections included 4 triple infections (2 fluA/hBoV/WUPyV, 1 adenovirus/hBoV/WUPyV, and 1 RSV/hBoV/WUPyV). Clinical data were available for 57 of the 62 WUPyV-positive NPA. A broad spectrum of both upper (45.6%) and lower (54.4%) respiratory tract diseases was observed. The latter included

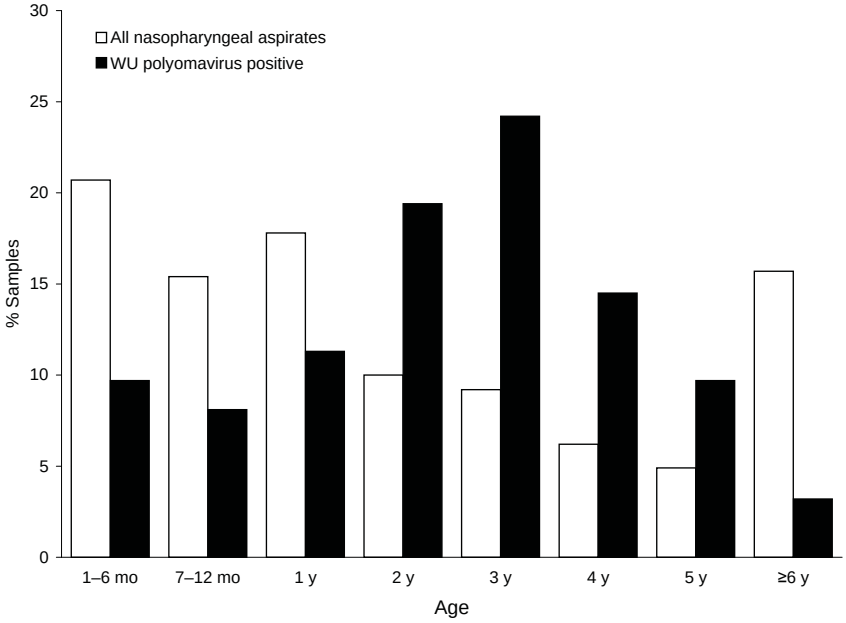

Figure. Age distribution of children with WU polyomavirus DNA–positive nasopharyngeal aspirates compared with the age distribution of the total study population.

bronchitis, wheezing bronchitis, and pneumonia.

In the context of the previous reports of WUPyV detection in Australia and North America (*3*), our data suggest a worldwide distribution of WUPyV. Most of the WUPyV-positive children were <4 years of age, and WUPyV DNA was rarely found in children >6 years of age. This age distribution is compatible with WUPyV infection occurring in day nurseries and kindergartens. In keeping with the findings of Gaynor et al. (*3*), we observed a high number of co-infections. The true number of co-infections in our study is probably higher than the reported 53.2% because we did not test for several respiratory pathogens, such as coronaviruses, rhinoviruses, enteroviruses, and the human metapneumovirus. Hypotheses to account for the detection of WUPyV in respiratory samples include the following: WUPyV is a persisting asymptomatic virus that is detected by chance, WUPyV is a persisting virus that is reactivated by an inflammatory process, or WUPyV is a predisposing or aggravating factor of respiratory diseases. Further studies are necessary to determine whether WUPyV is a human pathogen.

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Hepatitis E, Central African Republic

To the Editor: Outbreaks of hepatitis E virus (HEV) have been documented in many geographic regions and nonindustrialized countries (*1*–*3*); they have been primarily associated with fecal contamination of drinking water (*4*). In the Central African Republic (CAR), economic indicators (CAR ranks 172/177 countries on the 2006 United Nations Development Program Human Development Index), political instability, geographic situation, a deteriorating health network, and a very poor epidemiologic surveillance system all contribute to the country's epidemic susceptibility.

In July 2002, Ministry of Health (MoH) and Médecins sans Frontières (MSF) teams working in the Begoua Commune Health Center, north of CAR's capital Bangui, reported an increased number of patients from the Yembi I neighborhood who were showing signs of jaundice and extreme fatigue.

Patients suspected of having hepatitis E were defined as those with clinical jaundice (yellow discoloration of the sclera) and symptoms of malaise, anorexia, abdominal pain, arthralgia, and fever. Confirmed cases were those in which patients' serum samples were positive for HEV immunoglobulin (Ig) M or IgG.

Initially, 16 pairs of serum and stool samples were collected from jaundiced patients. Fecal samples were stored at –20°C and sent to the National Reference Center of Enterically Transmitted Hepatitis, Hospital Val de Grâce (Paris, France) for HEV marker testing; serum samples were tested at the Bangui Pasteur Institute for yellow fewer (YF) IgM by MAC-ELISA.

The HEV epidemic was confirmed by the detection of HEV markers: HEV IgG (Enzyme Immuno Assay, HEV, Abbott Laboratories, Abbott Park, IL, USA), HEV IgM (Abbott Laboratories), amplification of RNA (*5*), and the absence of YF IgM. The HEV genome was detected in 4 of the fecal samples. Genotyping and sequencing showed that one of these was genotype 1, prevalent in Africa; the others were related to genotype 2 (Mexico-like) (GenBank accession nos. DQ151640, DQ151640) (*5*,*6*).

Data suggest that the epidemic began in the Yembi I neighborhood, then spread to the rest of the Begoua commune and finally to Bangui or surrounding areas (Figure). Of 715 suspected HEV case-patients recorded in the MSF hospital between July 22 and October 25, 2002, 552 (77%) lived in the Begoua commune (271 in the Yembi I neighborhood). The attack rate for the Begoua commune (20,080 inhabitants) was 2.7%. Of 351 suspected case-patients serologically tested for IgG and IgM anti-HEV antibodies, 222 (63%) had IgM antibodies, including 5/16 pregnant women (2.3% of all confirmed cases). Most patients reported jaundice (97.5%) and choluria (95.1%);other reported symptoms

were nausea and vomiting (37.9%), dyspepsia (28.3%), and hepatomegaly and/or splenomegaly (26.4%). Four of the confirmed case-patients died, a case-fatality ratio (CFR) of 1.8%; one was a pregnant woman (CFR 20% for pregnant women group).

No significant differences were found among confirmed case-patients by sex or age-group. Seventy-seven (34.6%) had relatives with suspected HEV, and 163 (73.5%) had drunk untreated water from their own wells.

These epidemiologic findings suggest the water-borne nature of this outbreak. Environmental testing of water from 2 wells (before chlorination was implemented) showed the water to be unsafe to drink (i.e., heat-resistant coliforms and aerobic bacteria were present) (*7*).

The outbreak was not surprising because a 1995 survey in Bangui showed anti-HEV antibodies in 24% of patients tested (*8*), indicating endemic HEV. Our results for IgG-positive patients were similar (23.2% in men and 20.1% in women). As demonstrated during other outbreaks (*3*), we found no significant difference between the distribution of HEV-positive patients by age or sex, although most patients were males (58%) and young adults (71% of ages 14–45 years).

The observed CFR was similar to that in other reported HEV outbreaks, in which CFR varied from 1% to 4% (*9,10*), but it was as high as ≥30% in pregnant women (*9*). Deliveries during pregnancy months 6–8 in this outbreak highlight the need for close surveillance of pregnant women affected by this disease.

We recommended application of preventive measures, including water disinfection, safe disposal of excreta, community health education, and the strengthening of case management and disease surveillance. For the CAR, free access to a safe water supply and drugs was the only way to achieve these goals.

The number of HEV cases in the Yembi I neighborhood declined after the crisis team implemented hygienic and chlorination measures in the district, although the number of cases remained constant in other neighborhoods of the commune (Figure). Definite conclusions cannot be drawn from this finding. First, the MSF hospital was within the Begoua commune. Thus, patients from the rest of Bangui (outside the commune) only started arriving at the center for treatment after hearing about the hospital through broadcast messages. Second, a military coup d'état during epidemiologic week 43 prevented us from conducting further surveillance.

Our results agree with international data on HEV outbreaks in other nonindustrialized countries. However, studies to improve our understanding of this epidemic and to identify the main risk factors involved would be beneficial.

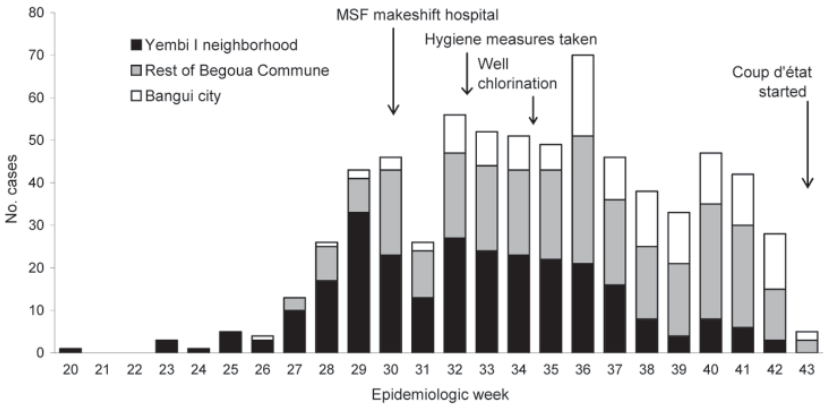

Figure. Suspected cases of hepatitis E virus in Begoua, Central African Republic, by neighborhood, weeks 20–43, 2002. MSF, Médicins sans Frontières.

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Rickettsia sibirica subsp. *mongolitimonae* Infection and Retinal Vasculitis

To the Editor: *Rickettsia sibirica* subsp. *mongolitimonae* is an intracellular bacterium that belongs to the species *R. sibirica* (*1*). To date, only 11 cases of infection with this bacterium have been reported (*2–6*). We report a case in a pregnant woman with ocular vasculitis.

A 20-year-old woman in the 10th week of her pregnancy was admitted in June 2005 to St. Eloi Hospital in Montpellier, France, with an 8-day history of fever, eschar, hemifacial edema, and headache. On examination the day of admission, she had a fever of 38.5°C, headache, and frontal eschar surrounded by an inflammatory halo. Painful retroauricular and cervical lymphadenopathies were noted. Results of a clinical examination were otherwise within normal limits. No tick bite was reported by the patient, although she had been walking a few days before in Camargue (southern France). Serologic results for *R. conorii*, *R. typhi*, *Brucella* spp., *Borrelia* spp., and *Coxiella burnetii* were negative.

One day after admission, she reported loss of vision (scotoma) in her right eye. She underwent a complete ophthalmic evaluation. Measurement of visual acuity and results of a slit-lamp examination were within normal limits, but a funduscopic examination showed a white retinal macular lesion that corresponded in a fluorescein angiograph to an area of retinal ischemia induced by vascular inflammation and subsequent occlusion (Figure). The following day, a rash with a few maculopapular elements developed, which involved only the palms of the hands and soles of the feet. Mediterranean spotted fever was suspected. Cyclines and fluoroquinolones were contraindicated because of her pregnancy, and the patient had a history of maculopapular rash after taking josamycin. She was treated with azithromycin, 500 mg/day for 10 days, under close surveillance. After 2 days of treatment, she was afebrile and the rash completely resolved. No obstetric complications occurred and she gave birth to a healthy boy at term. Two years later, the right scotoma remained unchanged.

Serologic tests for rickettsiosis were performed with an acute-phase serum sample and a convalescent-phase serum sample (1 month after onset of symptoms). Samples were sent to the World Health Organization Collaborative Center in Marseille for rickettsial reference and research. Immunoglobulin (Ig) G and IgM titers were estimated by using a microimmunofluorescence assay; results were negative. Culture of a skin biopsy specimen from the eschar showed negative results.

DNA was extracted from eschar biopsy specimen and used as template in a PCR with primers complementary to portions of the coding sequences of the rickettsial outer membrane protein A and citrate synthase genes as described (*5*). Nucleotide sequences of the PCR products were determined. All sequences shared 100% similar-

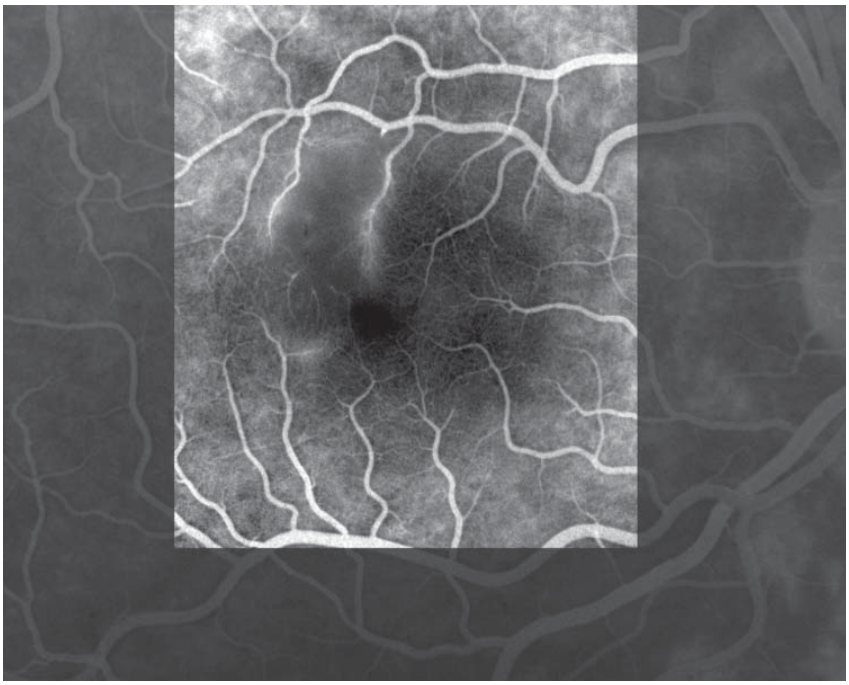

Figure. Fluorescein angiograph of the right eye of the patient showing retinal occlusive vasculitis with arteriolar leakage at late phase.

ity with *R. sibirica* subsp. *mongolotimonae* when compared with those in the GenBank database.

Infections caused by *R. sibirica* subsp. *mongolitimonae* have been reported as lymphangitis-associated rickettsiosis (*4*). Our case-patient had the clinical symptoms reported for this disease: fever, maculopapular rash, eschar, enlarged satellite lymph nodes, and lymphangitis. Seasonal occurrence of this disease in the spring is common and has been reported in 9 of 12 cases, including the case-patient reported here (*2*–*6*). A total of 75% of these *R. sibirica* subsp. *mongolitimonae* infections occurred in southern France; other cases have been recently reported in Greece (*5*), Portugal (*6*), and South Africa (*7*). However, the vector of *R. sibirica* subsp. *mongolitimonae* has not been identified (*7*). This rickettsia has been isolated from *Hyalomma asiaticum* ticks in Inner Mongolia, from *H. truncatum* in Niger (*8*), and from *H. anatolicum excavatum* in Greece (*5*). *Hyalomma* spp. ticks are suspected of being the vector and are widespread in Africa, southeastern Europe (including France), and Asia.

Rickettsiosis caused by *R. rickettsii* and *R. conorii* during pregnancy has been reported without risk for vertical transmission (*9*). First-line antimicrobial drugs used to treat rickettsial disease are cyclines and quinolones, but they are contraindicated during pregnancy. Chloramphenicol is an alternative drug for pregnant women but it is not available in France. Macrolides (azithromycin, clarithromycin, and josamycin) are effective against rickettsial disease and can be used safely during pregnancy.

No ocular complications were reported in the 11 previous cases of rickettsiosis caused by *R.* subsp. *mongolitimonae*. However, ocular lesions, including optic disk staining, white retinal lesions, retinal hemorrhages, multiple hypofluorescent choroidal dots, mild vitritis, and retinal vasculitis, have been described in patients with rickettsiosis caused by *R. conorii*, *R. rickettsii*, and *R. typhi* (*10*). Most of these posterior segment manifestations are usually asymptomatic in patients with acute Mediterranean spotted fever (*10*) and can be easily overlooked. Retinal vasculitis was reported in 45%–55% of the patients, but retinal artery occlusion secondary to vasculitis has been described in only 2 cases of infection with *R. conorii* and *R. rickettsii* (*10*) without details of clinical symptoms. Because ocular involvement could be asymptomatic and easily overlooked, an ophthalmic evaluation should be conducted when rickettsiosis is suspected.

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Rickettsia felis in Fleas, France

To the Editor: *Rickettsia felis* belongs to the spotted fever group of rickettsia. The pathogenic role of this intracellular Proteobacteria in humans has been reported in patients from the United States (Texas) (*1*), Mexico (*2*), Germany (*3*), Brazil, and France (*4*). *R. felis* is widely distributed, is associated with blood-sucking arthropods, and has been isolated from fleas in several countries (*5*).

To obtain new information about the distribution of *R. felis* in France and potential vectors/reservoirs of this emerging pathogen, 550 fleas were collected from 82 dogs and 91 cats in 7 widely distributed locations in France (Bordeaux, Toulouse, Cosnes-Cours sur Loire, Dijon, Moulins, Limoges, and Aix-en-Provence). Specimens were collected by combing, recorded, and stored at –20°C. Samples were shipped on dry ice to the entomologic laboratory of the Institute of Comparative Tropical Medicine and Parasitology in Munich, Germany, and species identification was performed by

using light microscopy and following the determination key of Hopkins and Rothschild (*6*). Because infestation levels varied (1–150 fleas/animal), we randomly analyzed 1–8 fleas (mean 3.4) from each host animal.

We homogenized fleas individually in 80 μL of phosphate-buffered saline by using 5-mm steel beads in a RETSCH Tissue Lyser Mixer Mill 300 (QIAGEN, Hilden, Germany). A total of 100 μL of ATL buffer and 20 μL of proteinase K (QIAGEN) were added, and the homogenate was incubated at 56°C in a thermomixer (Eppendorf, Hamburg, Germany) until the tissues were lysed. DNA was extracted from each flea by using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions (tissue protocol) and stored at –20°C until used in a PCR.

PCR amplification of rickettsial DNA was performed by using previously described oligonucleotide primer pairs Rp CS.877p/Rp CS.1258n targeting the citrate synthase (*gltA*) gene and, for the positive samples, Rr 190.70p/Rr 190.602n targeting the outer membrane protein A (*ompA*) gene (*7*). Amplification was conducted in 50-μL volumes that contained 5 μL of DNA, 30 μL of distilled water, 10 μL of 5× Taq buffer (Roche, Mannheim, Germany), 3 μL of 25 mmol/L $MgCl_2$ (Roche), 1 μL of 10 mmol/L deoxynucleotide triphosphates (Roche), 0.25 μL of each primer (100 μM), and 0.5 μL (5 U/mL) of Taq polymerase (Roche). Conditions for the *gltA* and *ompA* PCRs were as described by Bertolotti et al. (*8*). Negative and positive controls were included in all PCRs. All PCR products were separated by electrophoresis on 1.5% agarose gels at 100 V for 60 min and examined under UV light. For both genes, positive samples were purified by using the QIAquick PCR Purification Kit (QIAGEN) and sent for sequencing to the MWG Biotech Company (Martinried, Germany). Sequences were compared with those of previously characterized rickettsia in GenBank by using basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov) analysis.

Five species of fleas were identified: *Ctenocephalides felis* (500, 224 from dogs and 276 from cats), *C. canis* (37 from dogs), *Pulex irritans* (11 from dogs), *Spilopsyllus cuniculi* (1 from a cat), and *Archaeopsylla erinacei* (1 from a cat). Five dogs had mixed populations of fleas; 3 of these had *P. irritans* and *C. felis*, and 2 had *C. felis* and *C. canis*. One cat had *P. irritans* and *C. felis*, and another cat had *S. cuniculi* and *C. felis*. A total of 52 (19%) of the 272 fleas from dogs and 44 (16%) of the 278 fleas from cats were positive for both the *gltA* and *ompA* genes. Positive samples were obtained from all locations. Prevalence ranged from 6% (Dijon) to 43% (Toulouse) for dogs and from 3% (Moulins) to 37% (Bordeaux) for cats (Table). Of 550 fleas, 96 were positive for both genes (*gltA* and *ompA*) and 3 of 5 species of fleas were infected: 10 with *C. canis*, 85 with *C. felis*, and 1 with *A. erinacei*. All sequences matched *gltA* and *ompA* genes from *R. felis* (similarity 99%–100%).

Our investigation provides new information about distribution of *R. felis* and widespread flea infection with *R. felis* in France. A total of 88% of infected fleas were *C. felis*, but we found infected *C. canis* in Bordeaux and Toulouse and infected *A. erinacei* in Limoges. We report the presence of *R. felis* in *C. canis* and *A. erinacei* in France. *R. felis* in dog fleas in Uruguay and in hedgehog fleas in Algeria has been reported (*9*,*10*). Our findings indicate that these 2 flea species may be vectors of human *R. felis* rickettsiosis in France.

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Table. Prevalence of *Rickettsia felis* in fleas from dogs and cats, France*

Locality	Animal	No. animals	No. fleas	Flea species	No. (%) *gltA*+ *ompA*+
Aix-en-Provence	Dog	6	20	*Ctenocephalis felis*,† *C. canis*,† *Pulex irritans*	6 (30)
Bordeaux	Dog	14	67	*C. felis*,† *C. canis*, *P. irritans*	8 (12)
	Cat	11	38	*C. felis*†	14 (37)
Cosnes-Cours sur Loire	Dog	15	44	*C. felis*,† *C. canis*	7 (16)
	Cat	17	50	*C. felis*†	3 (6)
Dijon	Dog	6	18	*C. felis*,† *C. canis*	1 (17)
	Cat	1	3	*C. felis*†	1 (33)
Limoges	Dog	15	45	*C. felis*†	7 (16)
	Cat	21	61	*C. felis*,† *Archaeopsylla erinacei*†	11 (18)
Moulins	Dog	12	36	*C. felis*,† *C. canis*	5 (14)
	Cat	22	65	*C. felis*,† *Spilopsyllus cuniculi*	2 (3)
Toulouse	Dog	14	42	*C. felis*,† *C. canis*†	18 (43)
	Cat	19	61	*C. felis*†	13 (21)

**gltA*, citrate synthase A; *ompA*, outer membrane protein A.
†Species positive for *gltA* and *ompA*.

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Novel Nonstructural Protein 4 Genetic Group in Rotavirus of Porcine Origin

To the Editor: Infection with group A rotavirus is the main cause of acute gastroenteritis in infants and young children worldwide and in young animals of many species, including piglets. In recent years, several epidemiologic studies designed to monitor the appearance of novel or atypical rotavirus antigenic types have provided evidence for the increasing antigenic diversity of group A rotaviruses (*1*–*3*). In addition to the 2 rotavirus classification systems, VP7 (G) and VP4 (P) genes, the virus can also be classified on the basis of the nonstructural glycoprotein 4 (NSP4)–encoding gene. Sequence analyses of the NSP4 gene indicated the presence of at least 5 distinct genetic groups among human and animal rotaviruses, termed A to E (*1*,*4*,*5*). Among human rotaviruses, the diversity of NSP4 genes has been restricted mainly to genetic groups A and B; only a few human strains possess genetic group C. Conversely, all 5 NSP4 genetic groups (A–E) have been identified in rotaviruses of animal origins. To our knowledge, porcine rotaviruses (PoRVs) have been reported to belong only to NSP4 genetic group B (*1*).

During an epidemiologic survey of PoRV from June 2000 through July 2001, a total of 175 fecal specimens were collected from diarrheic piglets from 6 different farms in Chiang Mai Province, Thailand. Of these, 39 (22.3%) specimens were positive for group A rotavirus (*6*). A novel and unusual PoRV CMP034 strain was isolated from a 7-week-old piglet during this survey. Molecular genetic characterization showed that the CMP034 strain carried a novel P[27] genotype with a new lineage of G2-like rotavirus genotype (*7*). We performed a molecular analysis of the NSP4 gene of this strain in comparison with those of other NSP4 gene sequences available in the GenBank database.

The full-length of NSP4 gene was amplified by NSP4–1a and NSP4–2b primer pairs (*8*). The PCR amplicon was sequenced in both directions by using the BigDye Terminator Cycle Sequencing kit (PerkinElmer-Applied Biosystems, Inc., Foster City, CA, USA) on an automated sequencer (ABI 3100; PerkinElmer-Applied Biosystems, Inc.). The sequence of CMP034 was compared with those of reference strains available in the National Center for Biotechnology Information GenBank database by using BLAST (www.ncbi.nlm.nih.gov/blast). The NSP4 nucleotide sequence of the CMP034 strain was deposited in GenBank under accession no. DQ534017.

The complete NSP4 nucleotide sequence of PoRV CMP034 strain was 750 bp and contained a single long open reading frame coding for a protein of 175 aa. Comparative analysis of the CMP034 NSP4 sequence with those of the 5 representative established genetic groups (A–E) showed the highest sequence identity, at 92.6% nt and 96.9% aa levels, with 1 PoRV strain, P21–5 (*9*). However, CMP034 and P21–5 shared a low degree of sequence identity with other NSP4 genetic groups. The NSP4 sequence identities of the CMP034 and P21-5 strains ranged from 74% to 78% nt and 75%–79% aa levels with those of genetic group A; 77%–86% nt and 79%–86% aa levels with genetic group B; 69%–73% nt and 75%–78% aa levels with genetic group C; 62%–65% nt and 55%–60% aa levels with genetic group D; and only 43%–50% nt and 29%–33% aa levels with genetic group E. The phylogenetic tree confirmed that PoRV strains CMP034 and P21–5 were located exclusively in a separated branch, which was distantly related to the other 5 known NSP4 genetic groups (Figure). However, a bootstrap support for the separation of

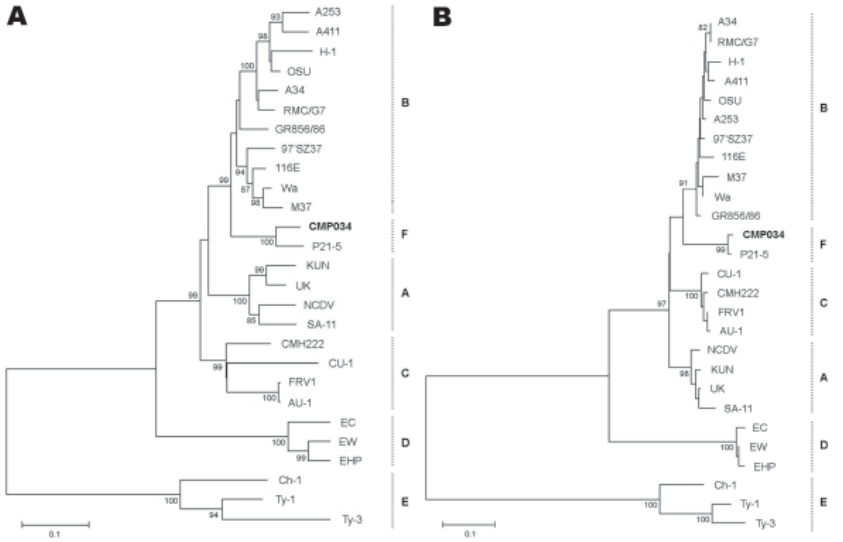

Figure. Phylogenetic analyses of the NSP4 nucleotide (A) and amino acid (B) sequences displaying the relationships between porcine rotavirus strain CMP034 (shown in **boldface**), P21–5, and other 5 known NSP4 genetic groups. Bootstrap values are shown at the branch nodes. Branch length for a 10% nucleotide difference is indicated at the bottom.

the gene into a separate lineage is very strong with nucleotide sequencing but weak by amino acid analysis in this phylogentic tree. Our finding indicates that PoRV strains CMP034 and P21–5 are likely a novel NSP4 genetic group and, therefore, tentatively proposed as a NSP4 genetic group F.

On the basis of the accumulated evidence of transmission of rotaviruses between pigs and other animal species, including humans, pigs are regarded as 1 potential reservoir for the emergence of unusual or novel strains of rotaviruses (*6*,*7*). In our study, the virus carried a novel NSP4 genetic group that has been isolated from a diarrheic piglet in Thailand. The NSP4 sequence analysis of our CMP034 strain revealed a PoRV strain closely related genetically to the NSP4 gene sequence of PoRV strain P21–5 isolated in Slovenia (*9*). PoRV strains CMP034 and P21–5 shared the same VP4 genotype as P[27] with over 90% aa sequence identity. The only difference observed between the 2 strains was that CMP034 belonged to the G2-like genotype whereas P21–5 belonged to G1 genotype. The relatedness between NSP4 sequences of strains CMP034 and P21–5 was confirmed by phylogenetic analysis, which showed that both CMP034 and P21–5 clustered closely together in a branch separated from those of other 5 NSP4 genetic groups. This finding suggests that NSP4 of PoRV strain CMP034 and P21–5 may have derived from the same ancestor. The isolation of 2 strains of rotaviruses with a close genetic relatedness of NSP4 gene from Thailand and Slovenia, 2 countries that are located in different continents, may indicate that this novel NSP4 genetic group has already been introduced into PoRVs worldwide. To verify this hypothesis, extensive epidemiologic surveillance of rotavirus in pigs may need to be conducted in several other regions of the world.

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PorB2/3 Protein Hybrid in *Neisseria meningitidis*

To the Editor: Class 2 and class 3 porin (PorB) proteins are the major proteins found in the outer membrane of *Neisseria meningitidis* (*1*); they function as porins, allowing the passage of small molecules through the outer membrane. PorB outer membrane proteins are transmembrane proteins with 8 predicted surface-exposed loops (I-VIII), which vary in length and in amino acid sequences. Several sequence analyses of these proteins have shown 4 regions with a high level of amino acid variability in loops I, V, VI, and VII (variable regions [VRs] 1–4) (*2*). The extensive antigenic variability of these proteins forms the basis of the *N. meningitidis* serotyping scheme (*3*,*4*). These 2 classes of proteins are mutually exclusive, and they are expressed by alternate alleles (*porB2* and *porB3*) at the *porB* locus (*1*).

All *N. meningitidis* strains received in the Spanish Reference Laboratory for *Neisseria* are routinely serotyped by whole-cell ELISA (*5*) with a set of monoclonal antibodies (MAbs) provided by the National Institute for Biological Standards and Control (South Mimms, UK) that includes the following serotypes: 1 (MN3C6B), 2a (5D4–5), 2b (MN2C3B), 4 (5DC4-C8G8), 14 (MN5C8C), 15 (8B5–5G9), and 21 (6B11F2B5). Those meningococci that appear as nonserotypeable (NT) are analyzed by sequencing the *porB* gene (*6*). In the case discussed here, in the sequencing of a NT strain, the *porB* gene showed an unusual sequence.

This strain, isolated in Spain during 2006, was recovered from the cerebrospinal fluid of a patient with meningococcal disease. The *porB* gene sequence shows VR1–4, which is exclusive of PorB3 protein, and VR2-Eb, VR3–2ab, and VR4-Cc, which are typical of PorB2 (GenBank accession no. EF094023). A comparison of this new sequence with the available *porB* sequences in the *Neisseria*.org database (http://neisseria.org/nm/typing/porB) enabled a more detailed analysis of the fragments corresponding to *porB3* and *porB2* found in this sequence. The fragment from nt 1 to 213 was identical to the *porB3–193* allelic variant (VR1–4, VR2-Aa, VR3–7, VR4–14b), and the second part, with nt 233–972 identical to *porB2–99* (VR1-Dc, VR2-Eb, VR3–2ab, VR4-Cc). The region of 214–232 nt is identical in the 3 variants. Therefore, this is a true hybrid molecule, which appears to have arisen from recombinational events between *porB2–99* and *porB3–193* alleles. In fact, this finding has prompted the inclusion of a new family called porB2/3 hybrid in the *Neisseria*.org database to facilitate the collection of this type of *porB* sequences.

The most likely origin of the *porB2/3* hybrid (4, Eb, 2ab, Cc) is the acquisition of DNA that encodes a VR1–4 sequence by a meningococcus with a *porB2–99* allelic variant. It is less likely that DNA encoding the *porB* VR2-Eb, VR3–2ab, and VR4-Cc sequences was acquired by a meningococcus with the *porB3–193* allelic variant because a longer fragment of DNA would have been transferred.

In spite of the presence of a VR1–4, which should be recognized by the set of MAbs used, this strain appeared as NT. A Western blot assay using MAb type 4 showed a good recognition epitope-MAb. Therefore, the failure of MAbs to identify this strain may have been due to the limited accessibility of the epitope because of the alteration of the PorB protein, which might be affecting its conformation. Once again, genetic characterization should be a preferred method over phenotypic characterization for typing meningococcal strains. Molecular characterization of NT strains in other laboratories might clarify the true frequency of this event.

Intragenic recombination between porin genes of the same allelic family is likely occurring in nature because mosaic gene structure has been reported in *porB* genes. However, *porB2/3* recombinants have never been previously found in the nature. Given the known ability of meningococci to be transformed by DNA from other strains, it is surprising that occurrence of genuine *porB2/3* hybrids has not yet been documented. There is only a report of naturally occurring gonococci expressing a hybrid *porB1a*/*porB1b* (*7*) (PorB1a and PorB1b gonococcus porins, as in meningococci, are encoded by 2 families of diverged alleles of the *porB* gene [*8*]). Gonoccocal strains expressing the recombinant *por* genes appear to be particularly susceptible to the bactericidal effect of human serum (*9*). A similar situation might happen in *N. meningitidis*, with a selective disadvantage in the invasive process of these hybrid strains, explaining the rarity of naturally occurring hybrids. By contrast, mechanisms like this are frequently used by meningococci to avoid the immune response against ordinary antigens. The balance between advantages and disadvantages at this level would show the true implications of this event.

This finding is relevant regardless of its frequency in nature. This report suggests how frequent the recombination events should occur among the meningococcal population: even theoretical mutually exclusive genes can produce hybrid variants; such knowledge is an important step in the development of future vaccines based on protein formulations.

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West Nile Virus in Birds, Argentina

To the Editor: West Nile virus (WNV), genus *Flavivirus*, family *Flaviviridae* has been rapidly dispersing through the Americas since its introduction in 1999 in New York (*1*). By 2004, serologic studies detected WNV-specific antibodies in birds and horses from Canada to northern South America (*2–4*). The first report of WNV activity in the Southern Cone of South America surfaced in April 2006, when 3 horses died in Argentina (*5*). However, established transmission foci in Argentina are unknown. We report evidence for the introduction and establishment of WNV in Argentina as early as January 2005.

Serum samples from free-ranging birds were collected from 5 locations in Argentina and screened for generic flavivirus antibodies by using a blocking ELISA with monoclonal antibody 6B6C-1 (*6*). Positive serum specimens were further characterized by plaque-reduction neutralization test (PRNT). We identified the etiologic agent responsible for the previous flavivirus infection by using the following criteria: 80% neutralization of reference virus (WNV NY99-4132 or an Argentinean strain of St. Louis encephalitis virus [SLEV CbaAr4005]) in serum diluted at least 1:40 and 4-fold greater titer compared with the other virus.

Overall, 474 (25.6%) of 1,845 serum specimens from 117 bird species collected from January to June 2006 tested positive when using the blocking ELISA; 30% inhibition was the threshold for a positive test. SLEV infections were confirmed in 105 birds by PRNT; WNV infections were confirmed in 43 birds. Anti-WNV antibody titers ranged from 40 to 2,560 in birds collected as early as January 2005 in Córdoba City and as late as June 2006 in Mar Chiquita (Table). Recent WNV activity was indicated by seroconversion in 3 banded rufous hornero in Córdoba City between January and March 2005. Although 1.5% of 659 serum samples were positive for SLEV, no WNV infection was detected in free-ranging birds collected in 2004. After 2004, WNV activity was detected in all 5 sampling locations and in a variety of ecosystems: Córdoba, periurban thorn forest (1.1%, 6/543); Mar Chiquita, thorn forest (5.1%, 16/313); Monte Alto, semi-dry chaco forest (9.8%, 8/82); Montecristo, cropland (9.5%, 2/21) and San Miguel de Tucumán, periurban yungas foothills (4.9%, 12/227).

In 2006, WNV was isolated from equines in Buenos Aires province (*5*). WNV transmission to resident birds collected further north in Córdoba, Chaco, and Tucumán provinces was detected in 2005 and 2006. Our data suggest that WNV was introduced into Argentina before 2005 and maintained naturally in enzootic foci where numerous bird species from many families were exposed. Presumably, as in North America, locally abundant passerine birds such as turdids (thrushes) are amplifying hosts. If common species of the *Furnariidae* (a family absent from temperate North America) prove to be competent hosts, they could play an important role in WNV transmission in Argentina because of their frequent exposure to WNV. Twelve (12.5%) of 96 *F. ruffus* sampled in 2005 and 2006 tested positive.

How WNV reached Argentina may never be known. Dispersal by migrating birds is a popular hypothesis, although relatively few North American breeding

Table. Prevalence of West Nile virus–neutralizing antibodies among birds grouped by taxonomic family, sampled in Chaco, Córdoba, and Tucumán Provinces, Argentina, 2004–2006*

Bird family	No. positive	No. tested	% Positive (95% CI)	Range of $PRNT_{80}$ titer†
Cardinalidae	2	54	3.7 (1.0–12.5)	80–160
Columbidae	4	270	1.5 (0.6–3.8)	80–1,280
Dendrocolaptidae	4	17	23.5 (9.6–47.3)	320–2,560
Falconidae	3	5	60.0 (23.1–88.2)	320–2,560
Furnariidae	12	201	6.0 (3.4–10.1)	80–1,280
Icteridae	3	137	2.2 (0.7–6.2)	40–320
Passeridae	1	87	1.1 (0.2–6.2)	40
Phasianidae	2	8	25.0 (7.1–59.1)	320
Polioptilidae	2	7	28.6 (8.2–64.1)	80–640
Troglodytidae	1	17	5.9 (1.0–27.0)	80
Turdidae	8	132	6.1 (3.1–11.5)	40–1,280
Tyrannidae	1	370	0.3 (0.05–1.5)	160

*Most of these families are of the order Passeriformes except for *Falconidae* (Falconiformes), *Phasianidae* (Galliformes), and *Columbidae* (Columbiformes). CI, confidence internal, determined by the Wilson score method for binomial proportions, without continuity correction.
†PRNT, plaque-reduction neutralization test. Titers are expressed as inverse of dilution.

birds migrate to Argentina, and austral migrants number fewer than boreal migrants. Komar and Clark (*2*) suggested that bird species in the order Charadriiformes, such as shorebirds and terns, are candidates for carrying WNV from North America to South America due to long lasting high-level viremias, occasional persistent infectious viral loads in skin, and direct, long-distance flights. WNV spread southward from the United States to northern South America between 1999 and 2004 following a stepping stone pattern, consistent with spread by birds. Moreover, introduction of WNV into Argentina by migratory birds could explain the presence of the virus in many places in a brief period. However, for migratory birds (211 serum samples tested) in this study, serologic test results were negative.

The high titers of WNV-reactive antibody are strongly indicative of WNV infections. Overall, 216 serum specimens reacted by PRNT test against SLEV, WNV or both at titers ≥20. Sixty-eight serum samples remain unidentified. The large number of unidentified flavivirus-positive samples detected by PRNT, ELISA, or both (148/474) could be due to 1) false positives; 2) cross-reactions between WNV- and SLEV-reactive antibodies that prevented definitive diagnosis by PRNT; 3) cross-reactive antibody and multiple, heterologous flavivirus infections; 4) previous infections by both WNV and SLEV; and/or 5) presence of other flaviviruses circulating in Argentina. SLEV is endemic throughout Argentina and, like WNV, belongs to the Japanese encephalitis virus serocomplex. Hemagglutination-inhibiting antibodies against several Brazilian flaviviruses (e.g., Bussuquara, Ilheus, Rocio viruses) have been reported in the neotropical region of extreme northern Argentina (*7*), but these viruses have not been isolated in Argentina.

Our serologic data suggest that WNV has established itself in 4 ecologic regions in Argentina in a brief period. Additional studies are needed to define the reservoir hosts and vectors of WNV in Argentina, and most importantly, to define the public health risk this virus represents.

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Clostridium difficile Surveillance Trends, Saxony, Germany

To the Editor: Vonberg et al. (*1*) recently commented on the increase of *Clostridium difficile* seen in US hospitals by using discharge diagnoses and confirmed the observation from the United States (*2*) with hospital discharge data from Germany during 2000 through 2004. *C. difficile* ribotype 027 has recently been isolated in Germany (*3*). We further contribute to the assessment of *C. difficile* as an emerging threat by looking at population surveillance data.

C. difficile is not a federal notifiable disease in Germany, which limits our ability to analyze national surveillance trends. However, in 2002 the state of Saxony implemented additional mandatory surveillance of community- and hospital-acquired infectious enteritis caused by laboratory-confirmed *C. difficile*.

To check for an increase in notifications due to reporting bias of gastroenteric diseases, we compared the quarterly incidence data from 2002 through 2006 with data on *Salmonella* spp. infections (usually reported by local general practitioners) and rotavirus and norovirus infections (both usually reported by clinics). The potential problem of reporting bias for gastroenteric diseases has been addressed recently (*4*). Information about age and sex of *C. difficile* patients was available for 2006 only.

Quarterly incidences for *C. difficile* in Saxony were from 1.7–3.8 per 100,000 population in 2002 and 2003 and continued to increase to 14.8 cases per 100,000 population in 2006 (Figure). This constitutes a 6-fold increase of the yearly average of *C. difficile* incidence rates between 2002 and 2006. The third quarter of 2005 experienced a sharp drop that could not be explained retrospectively and might have resulted from transition to new procedures for data collection and management.

Gastroenteric infections showed clear seasonality with a slightly decreasing yearly trend for *Salmonella* spp. and seasonal values from 13.8 cases per 100,000 in winter to summer peaks of 56.8. Rotavirus infections displayed an even stronger seasonality, with values from 7.0 cases per 100,000 in summer to winter peaks of 140.3. Norovirus infections peaked again during winter, at 137.2 cases per 100,000 but had as few as 11.0 cases per 100,000 during summer. Notification does not suggest reporting bias of gastroenteric infections.

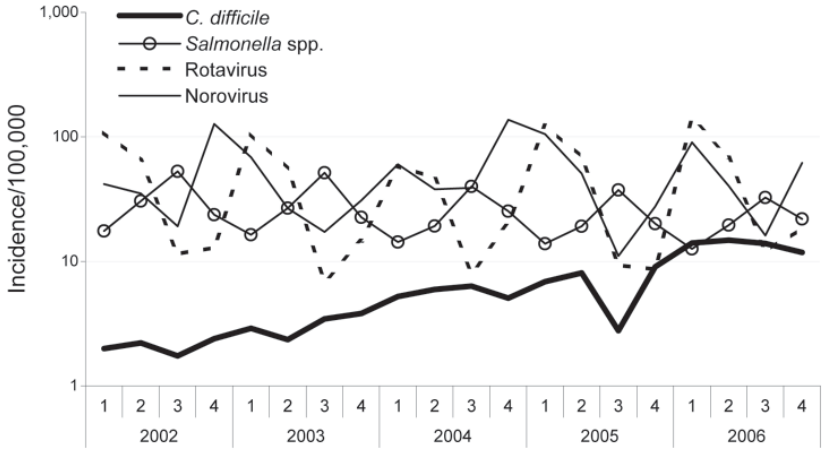

Figure. Quarterly incidence per 100,000 population of *Clostridium difficile* infections compared with gastroenteric infections caused by *Salmonella* spp., rotaviruses, and noroviruses in Saxony, Germany, 2002–2006. Note the log scale on the y axis.

Elderly persons, i.e., those ≥65 years of age, were affected most by *C. difficile* infections; this age group accounted for 1,506 (65%) of all cases (n = 2,306) in 2006. The 45- to 64-year age group had the next highest number of cases, 451 (20%). Men and women were affected equally in the different age groups; slightly more women (n = 805) than men (n = 701) with *C. difficile* infection were ≥65 years of age.

According to state and local health departments, there were no major health campaigns since 2004 that might have selectively increased awareness for *C. difficile* notification. Our results show a continuous increase of cases that even reaches seasonal notification levels of *Salmonella* spp. and rotavirus infections, but the increase is difficult to explain entirely by changes in reporting behavior. We emphasize the role of individual German states in setting additional surveillance targets for public health. Given the epidemic potential and the severity of the disease, especially among the elderly, surveillance of *C. difficile* should be introduced throughout Germany along with enhanced prevention and treatment strategies (*5*).

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Travel Medicine: Tales Behind the Science

Annelies Wilder-Smith, Marc Shaw, and Eric Schwartz, editors

Elsevier Science, New York, New York, USA, 2007
ISBN: 978-0-08-045359-0
Pages: 333; Price: US $42.80

This book is a compilation of 40 essays written by many of the most recognized names in the field of travel medicine. It is divided into 9 sections with such topics as the history of travel medicine, vaccines, travel medicine research, pilgrimages, and even space travel. Photographs, tables, and charts enhance the reader's interest, especially when one spots a familiar person or place.

The styles range from the didactic, to short story to poetry, and the mood ranges from the humor of Jay Keystone's "Ten Commandments" to the stark reality of Marc Shaw's "Amazonas Adventure." One cannot help but chuckle at Charles Ericsson's description of diarrhea research or laugh outright at Steve Toovey's "Woman Atop the Crocodile," and Nancy Piper Jenks' account of undocumented migrants may bring the reader to tears.

This is not a formal textbook of travel medicine, but much can be learned from it. Although not a history text, the book is replete with fascinating accounts of medical history. One learns such things as the origin of the word "quarantine," the complexities of preparing a certification examination, and the sheer terror of being on the front lines of an epidemic of severe acute respiratory syndrome. In short, the volume explains why things are the way they are in travel medicine and why this new discipline has, of necessity, become a separate specialty.

The essays need not be read in the order presented, but surprisingly, some of the topics that seem least interesting turn out to be the most fascinating. Much of the book reads like a medical detective story; other parts read like a medical journal but the writing is more compelling.

If I had to produce a criticism for the book, it would be simply that the publisher has picked a size of print that is almost too small for my presbyopic eyes. Overall, the book is a fascinating read, and one can only hope that future editions will be forthcoming.

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Coronaviruses: Molecular and Cellular Biology

Volker Thiel, editor

Caister Academic Press, Norfolk, UK, 2007
ISBN: 978-1-904455-16-5
Pages: 350; Price: US $300.00

Coronaviruses are a group of single-stranded RNA viruses that mainly cause enteric and respiratory diseases in infected hosts. Before 2002, coronaviruses were known as important veterinary pathogens, as well as a cause of the common cold in humans. In 2002–2003, with the advent of

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the outbreak of severe acute respiratory syndrome (SARS), this picture changed. SARS was quickly shown to be caused by a novel coronavirus, and the ensuing explosion of research on coronaviruses is reflected in this new book.

This multi-authored book contains 16 chapters and is organized into 2 sections. The first section of 7 chapters covers most aspects of coronavirus replication, from virus binding and entry into the cell to genome packaging. When appropriate, these chapters also draw on recent work with the closely related arteriviruses. Each chapter generally offers excellent and balanced reviews of the coronavirus literature through 2006, with a few references from 2007. The second section of 9 chapters discusses various aspects of the host-pathogen interface of several coronaviruses; the major focus is the SARS coronavirus, although the human coronavirus NL63 and murine, feline, and avian coronaviruses are also covered.

This book provides a one-stop entry into current thinking in the field. For those unfamiliar with coronaviruses, the first section offers a current view of how these viruses replicate. Two areas that are not as well represented in this section are effects of coronavirus infection on cellular processes, such as the cell cycle, apoptosis, and other signaling pathways, and protein trafficking, virus assembly, and release. Separate chapters on these areas would have strengthened the book. In some ways, the second section of the book is not as satisfying. The 3 chapters on SARS coronavirus and the chapter on human coronavirus vaccine development have introductory sections that are somewhat repetitive. Including chapters on transmissible gastroenteritis virus and porcine respiratory coronavirus would also have been beneficial. That said, the chapters on SARS and the avian, murine, and feline coronaviruses are excellent.

I heartily recommend that this book be placed in the library of every laboratory that is working on this fascinating group of viruses. It will be particularly valuable to newcomers to the field by providing a single entry point to recent thinking about these agents.

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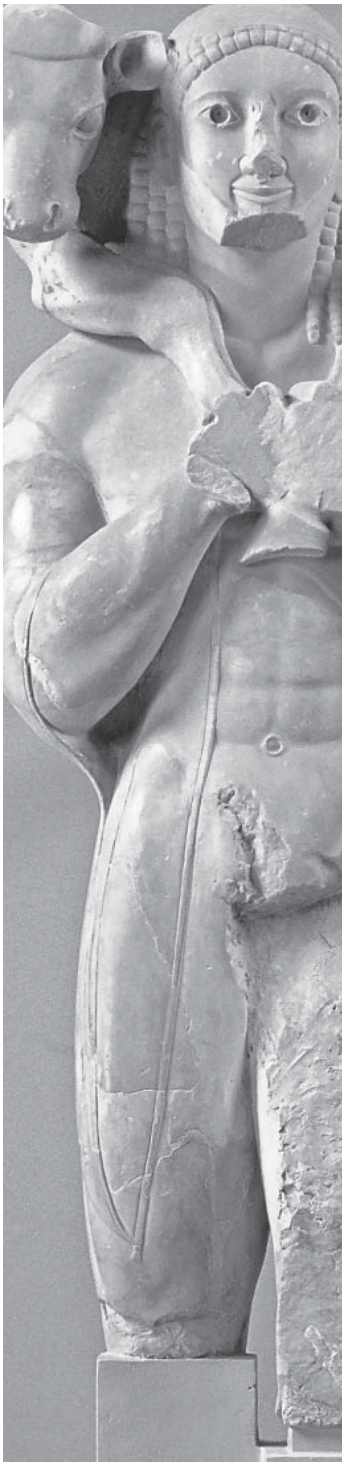
ANOTHER DIMENSION

The CAT Scan

Ronald O. Valdiserri

I enter your portal through a scrim of invisible rays,
beads of energy outnumbering my regrets.
Supine on a cold bed, part supplicant, part sacrifice.
Like all captives, fearing judgment.
Woosh, then comes the warm tingle of the dye,
everywhere at once.
It feels like a cleansing…
washing away debris, debt, equivocation.
"no," silently, I correct myself,
acknowledging the iodine's more melancholy assignment:
building a luminous marquee around 56 years of imperfection and wear.
And the lights blink yellow.

Dr Valdiserri is chief public health consultant in the Office of Public Health and Environmental Hazards at the US Department of Veterans Affairs. Prior to joining the VA in 2006, Dr. Valdiserri served as the deputy director of the National Center for HIV, STD, and TB Prevention at the US Centers for Disease Control and Prevention.



Moschophoros (Calf-Bearer) (detail) attributed to Phaidimos. Statue of the patriot Romvos offering sacrificial calf to Athena.

c. 570 BCE. Marble. Height 165 cm. No. 624 Acropolis Museum, Athens, Greece

"In Dreams Begin Responsibilities"

—William Butler Yeats

Polyxeni Potter*

"I woke with this marble head in my hands; / It exhausts my elbows and I don't know where to put it down. / It was falling into the dream as I was coming out of the dream / So our life became one and it will be very difficult for it to separate again," wrote George Seferis about his relationship with art from antiquity (*1*). Traversing the edges of time has long been the domain of artists and poets, who view history as a continuous process not to be fragmented and labeled "ancient" as if somehow interrupted or expired (*2*).

"The art of [marble] sculpture is much older than that of painting or bronze statuary," wrote Pliny the Elder (23–79 CE) (*3*). Early sculptors worked on marble with point chisels, punches, and stone abrasives. Repeated vertical blows shattered crystals deep into the stone, altering the outer gloss. Because statues were painted, the opaque surface benefited pigment application. On the Acropolis, the first marble statues appeared more than two thousand years ago (*4*). They were votives, mostly maidens called *kores* but also young men, *kouri*. Some were inscribed with the names of artists; others, with dedications. They represented the donor or a deity, a renowned athlete, or the deceased if intended for a gravesite. They dominated art of the archaic period (750–500 BCE). Thousands have been excavated from various sites.

Neither gods nor mortals, *kores* and *kouri* embodied physical perfection accessible to both. They were free-standing, the earliest such examples of large stone images of the human form in the history of art (*5*). Their arms were separated from the torso, the legs from each other. Tense and filled with life, they had various faces and expressions, their individuality foreshadowing portraiture. Their large eyes stared directly ahead, and they were injected with emotion, the stylized "archaic smile," signifying not happiness but emerging humanity. They wore flowing garments, carefully delineated, and appeared refreshed and carefree, as if suddenly become aware of themselves.

The best-known of these figures, Moschophoros (calf-bearer), on this month's cover, represented the donor, a nobleman named Romvos as inscribed on the base. The figure, found in fragments on the grounds of the Acropolis near the sanctuary of the Temple of Athena, has none of the masklike quality of earlier *kouri*. Though he has their usual left-foot-forward stance and stylized tufted hair, Moschophoros is not a youth but a mature man with a beard. His fitted cloak was likely painted in vivid colors as were the lips and hair. "The hollow eyes ... once held inlays of semi-precious stones (mother-of-pearl, gray agate, and lapis lazuli) that would have given the face a strikingly realistic appearance" (*6*).

Romvos is carrying an animal for sacrifice on the altar of Goddess Athena, a formidable fixture of the Hellenic pantheon known for its temperamental deities and countless demigods and their descendents. Their origins and relationships with humans were fodder for myths and art through sculpture and elaborate iconography. Gods gave gifts and favors. Humans offered votives as thanks, atonement, entreaty, or worship.

Sacrifice (from sacrificium [sacred] + facere [make] = to make sacred) was a central part of religious practice during festivals and feast days. The ritual was performed in well-defined space within a temple sanctuary. Some feasts were Pan-Hellenic and included processions and athletic competitions. "There are sanctuaries of Hermes Kriophoros," wrote Pausanias, describing the city of Tanagra. "... Hermes averted a

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pestilence from the city by carrying a ram round the walls; to commemorate this Calamis made an image of Hermes carrying a ram upon his shoulders. Whichever of the youths is judged to be the most handsome goes round the walls at the feast carrying a lamb on his shoulders" (*7*).

Homer mentions sacrifice "of bulls, of goats" in the Iliad and of "sleek black bulls to Poseidon, god of the sea-blue mane who shakes the earth," in the Odyssey. The most common offering was the sheep, goat, or pig, but the ox and bull were also used, depending on the occasion. Animals were selected for their physical perfection, their horns gilded and adorned with ribbons and garlands. As the animal walked toward the altar, barley was thrown in its path to entice it and water sprinkled on its head, causing it to nod as in agreement with the proceedings. The crowd was silent, then sorrowful, acknowledging the sacrifice. The ritual turned into a feast, "while the people tasted the innards, burned the thighbones for the god" (Odyssey, Book III).

The contradictions inherent in religious sacrifice did not elude ritual participants, who ate little meat outside these religious feasts. The rituals may have expressed their uneasiness at killing animals for food and to appease the gods. Their ambivalence continued during the classical period, when even large domestic animals sustainable only in small numbers were used. "Our ancestors handed down to us the most powerful and prosperous community ... by performing the prescribed sacrifices," wrote Athens orator Lysias, defending the practice. "It is therefore proper for us to offer the same ... if only for the sake of the success which has resulted from those rites" (*8*).

Moschophoros stuns for its ability to bring to life eons after its creation a moment of connection. The human face, wearing a smile, the single most appealing adornment then and now, is framed by the surrendered animal. The marble seems to melt in the calf's unparalleled fragility and tenderness. Locked in a secure embrace, human and animal take a step together, an ear touching, a tail relaxed.

"My pawing over the ancients and semi-ancients," wrote Ezra Pound, "has been one long struggle to find out what has been done, once for all, better than it can ever be done again, and to find out what remains for us to do" (*9*). Moschophoros captured the primeval ease between man and calf. What remains to do? For us the challenge is to get beyond the sacrifice. Whether to appease the gods or stop BSE, charred remains of cattle and other animals betray limited success in our symbiotic relationship. Increased animal translocation and ecologic transformation add to the intrigue, along with microbial changes now seen at the molecular level.

Moschophoros is not ancient. The statue exists in the present. It can be touched, viewed, and examined for universal meaning. Resilient and unchanged, it defies death. And like other marvels from antiquity, it takes the initiative in speaking to us. "The statues are not the ruins," wrote Seferis, "—we are the ruins" (*1*).

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Erratum: Vol. 14, No. 2

In the article "Molecular Typing of Australian *Scedosporium* Isolates Showing Genetic Variability and Numerous *S. aurantiacum*" by L. Delhaes et al., Figure 1, p. 286, contained errors. Profile C should have referred to lanes 7–12, and Profile B should have referred to lanes 13–18. The correct version of the figure is available from www.cdc.gov/eid/content/14/2/282-G1.htm.

We regret any confusion this error may have caused.

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Scale-up of Multidrug-Resistant Tuberculosis Laboratory Services, Peru

Declining Artesunate-Mefloquine Efficacy, Cambodia–Thailand Border

Community-onset Staphylococcal Disease, England

Increasing Hospital Admissions for Pneumonia, England

Transmission of Avian Influenza Virus (H3N2) to Dogs

Increasing Incidence of Listeriosis in France and Other European Countries

Efficacy of Adulticide for Control of West Nile Virus, California, 2005

Shiga Toxin–Producing *Escherichia coli* Infections in Children, Argentina

Increase in West Nile Neuroinvasive Disease after Hurricane Katrina

New Saffold Cardioviruses in 3 Children, Canada

Novel Poxvirus in Endangered Red Colobus Monkeys, Western Uganda

Social Support and Response to AIDS and Severe Acute Respiratory Syndrome

Acute Encephalitis Caused by Intrafamilial Transmission of Enterovirus 71 in Adult

Spread of *Streptococcus suis* Sequence Type 7, China

Morbillivirus and Pilot Whale Deaths, Mediterranean Sea

Cholera Outbreaks, Rural Bangladesh

Lakes as Source of Cholera Outbreaks, Democratic Republic of Congo

Rickettsia slovaca, *Dermacentor marginatus*, and Tick-borne Lymphadenopathy, Tuscany, Italy

Sandfly Fever Sicilian Virus, Algeria

Bacteremia Caused by Group G Streptococci, Taiwan

Complete list of articles in the May issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 5–8, 2008
Society for Healthcare Epidemiology of America (SHEA) 18th Annual Scientific Meeting
Buena Vista Palace
Orlando, FL, USA
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

April 28–May 2, 2008
National Biosafety and Biocontainment Training Program
Kansas State University Biosecurity Research Institute
Manhattan, Kansas, USA
http://www.nbbtp.org

May 5–7, 2008
Eleventh Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org/conferences/vaccine08

June 19–22, 2008
13th International Congress on Infectious Diseases
Kuala Lumpur, Malaysia
http://www.isid.org

June 24–27, 2008
ANAEROBE 2008
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Marriott Hotel
Long Beach, CA, USA
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Article Title: β-Herpesviruses in Febrile Children with Cancer

CME Questions

1. Which of the following statements about β-herpesviruses is *most* accurate?

A. Infection is not common until late adolescence

B. Infection with cytomegalovirus (CMV) promotes symptoms similar to mononucleosis

C. Human herpesvirus (HHV)-6B is the cause of fifth disease

D. HHV-7 is the virus responsible for most cases of roseola

2. Which of the following β-herpesviruses were detected in the patient cohort of the current study?

A. HHV-6B and HHV-6A

B. CMV and HHV-7

C. HHV-6B and CMV

D. HHV-6A and HHV-7

3. Which of the following statements about infection data in the current study is *most* accurate?

A. The etiology of most patients' fever was discovered during hospitalization

B. Infection with β-herpesviruses occurred at a higher frequency among children with cancer compared with those with solid organ transplant

C. Fever was generally higher among children infected with β-herpesviruses

D. HHV-6B infection was likely a reactivation of previous infection among cancer patients

4. Which cancer factors promoted infection with HHV-6B in the current study?

A. Solid organ tumor and over 6 months since the initiation of immune suppression

B. Solid organ tumor and less than 6 months since the initiation of immune suppression

C. Leukemia and less than 6 months since the initiation of immune suppression

D. Leukemia and more than 6 months since the initiation of immune suppression

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

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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.

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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.

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   - 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

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   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
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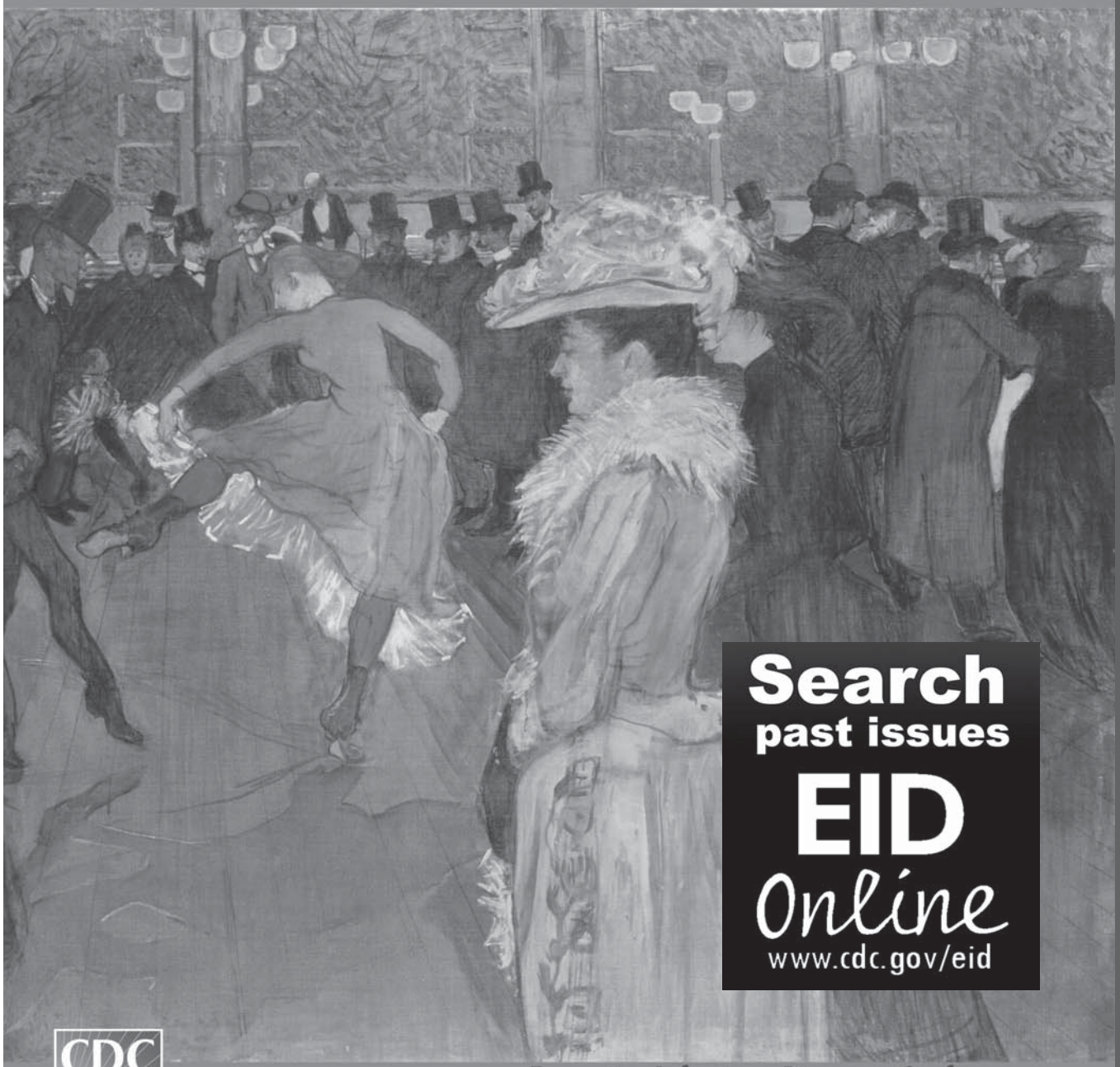

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Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

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.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

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.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

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.

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.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.