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On the Cover

Thomas Eakins (1844–1916).
John Biglin in a Single Scull (1874).
Oil on canvas (61.9 cm x 40.6 cm).
Yale University Art Gallery.
Whitney Collections of Sporting Art, given in memory of Harry Payne Whitney, BA 1894, and Payne Whitney, BA 1898, by Francis P. Garvan, BA 1897, MA (Hon.) 1922

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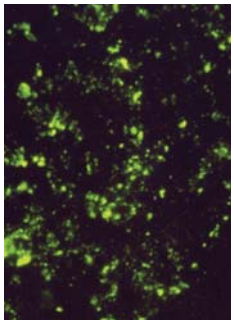

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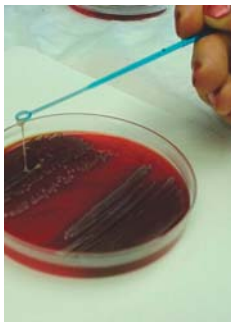

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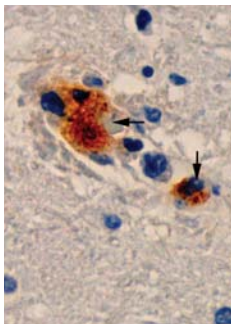

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Brazilian Vaccinia Viruses and Their Origins

Giliane S. Trindade,*[1] Ginny L. Emerson,*[1] Darin S. Carroll,* Erna G. Kroon,† and Inger K. Damon*

Although the World Health Organization (WHO) declared global smallpox eradicated in 1980, concerns over emergent poxvirus infections have increased. Most poxvirus infections are zoonotic; exploring their genetic diversity will illuminate the genetic and evolutionary aspects of poxvirus infections, ecology, and epidemiology. In recent decades, several strains of the orthopoxvirus vaccinia virus (VACV) have been isolated throughout Brazil, including genetically distinct isolates within the same outbreak. To further investigate the diversity and origins of these viruses, we analyzed molecular data from 8 Brazilian VACV isolates and compared several genes involved in virus structure and pathogenicity. Genetic variation among isolates suggests that ancestral Brazilian VACVs existed before the beginning of the WHO smallpox eradication vaccination campaigns and that these viruses continue to circulate.

In 1980, the World Health Organization (WHO), after a massive vaccination program, announced the eradication of smallpox, the contagious and deadly disease caused by variola virus (VARV). This program used live vaccinia virus (VACV), a virus from the same genus, *Orthopoxvirus*, which shares a high degree of immunologic cross-reactivity with VARV. Recent reports of cowpox virus (CPXV) infections in Europe, monkeypox virus (MPXV) outbreaks in Africa and the United States, and the surprising emergence of VACV in Brazil highlight the need for continued research into the ecology, epidemiology, origin, and evolution of these viruses (*1*).

The known history of VACV species imported to Brazil dates back to 1804, when human vaccine arrived at a port in Bahia State on the arms of slaves returning to Brazil from Lisbon, Portugal (*2*) (Figure 1). From Bahia State the slaves were sent south to Rio de Janeiro State, possibly passing the vaccine to local people as they made their way through the region. Indeed, from 1804 to 1887, the Brazilian population (including slaves and other people living in the countryside) was vaccinated in this manner, arm to arm. In some cities, vaccination was obligatory, beginning in 1832. In 1887, the first animal vaccine produced in calves was imported in flasks to the vaccine institute in Rio de Janeiro (now Oswaldo Cruz Institute) from the Chambon Institute in Paris, France (*2*). The vaccine was then distributed to other states across Brazil, including Minas Gerais, Espirito Santo, São Paulo, Mato Grosso, Rio Grande do Sul, and Pernambuco (*2*). During this time, between 1887 and 1895, vaccine institutes were established in these states (*2*).

Published accounts of Brazilian vaccinia-like viruses isolated from sentinel mice and recent outbreaks on dairy farms affecting cattle and their handlers suggest the once-emergent disease has now become endemic (*4–13*). We combined historical information and published data to develop insights into the possible origin(s) of the Brazilian VACV (BRZ-VACV) now established in Brazil. Our goal was to determine whether BRZ-VACV represents an escaped vaccine strain, an autochthonous orthopoxvirus, or both.

Methods

We examined the sequence diversity of 8 geographically and temporally variable BRZ-VACV isolates. We compared molecular sequence data from 3 genes and a variable region of the poxvirus genome (Table 1) among BRZ-VACV isolates, available vaccine strains related to those used during the eradication campaign in Brazil, and other VACVs isolated from domestic animals (including endemic buffalopox virus in India [*15–17*] and horsepox virus [HSPV] from Asia [*18*]).

Sequences were collected from GenBank (Tables 1 and 2). Some isolates had limited sequence data for the 4 genes examined. Although all 4 genes are not available from Lister (B19R gene is not present in the Lister ge-

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[1]These authors contributed equally to this work.

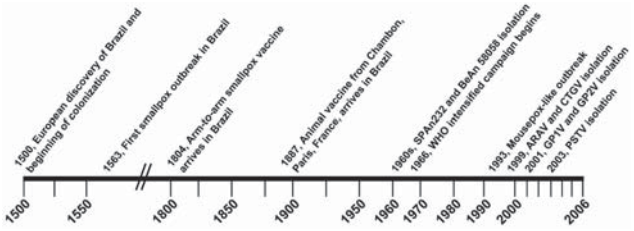

Figure 1. Timeline of events regarding the introduction and circulation of orthopoxviruses in Brazil (*2,3*). Double slashes indicate a gap in the timeline.

nome), the Lister isolate was included because of its importance in the history of vaccination in Brazil (*3*,*6*). Sequences from 3 genes were manually aligned and trimmed to include only the regions available for all isolates (VACV-COP A56R 161210–162095, E3L 51465–50929, B19R 178242–179173). Nucleotide identities were calculated by using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html) (online Technical Appendix, available from www.cdc.gov/EID/content/13/7/965-Techapp.pdf). PAUP* version 4 (Sinauer Associates; Sunderland, MA, USA) and MrBayes 3.1 (http://mrbayes.csit.fsu.edu) were used to analyze a matrix of the 3 concatenated genes (*19*,*20*). Parsimony analysis was performed on 1,000 random addition replicates. Appropriate models of sequence evolution were determined by Modeltest (*21*) with the Akaike information criterion (*22*,*23*). The identified model was then applied to maximum likelihood and Bayesian analyses. In MrBayes, the Markov chain Monte Carlo searches explored 4 chains for 1 million generations, sampling every 100 generations. Sample points during the first 50,000 generations were discarded as burn-in, before which the chain reached stationarity (fixed condition). To account for any considerable rate variation between genes, a second Bayesian analysis was run wherein each gene was partitioned and allowed to vary independently (MrBayes command: prset ratepr = variable). DNA sequence data for the fourth coding region, rpo132-ATI-p4c-A27 (A-type inclusion body gene), were not included in the phylogenetic analysis; however, published digestion profiles from relevant strains were compared.

Results

The resultant phylogenetic tree (Figure 2A) depicts several highly supported clusters including monophyletic VACV, MPXV, VARV, and camelpox virus (CMLV) assemblages. VARV and CMLV isolates are depicted as sister taxa with high support. Brazilian isolates fall into 2 well-supported monophyletic groups. Group 1 comprises Araçatuba virus (ARAV), Cantagalo virus (CTGV), Guarani P2 virus (GP2V), and Passatempo virus (PSTV); group 2 comprises GP1V, Belo Horizonte virus (VBH), BeAn 58058 virus (BAV), SPAn232 virus (SAV), and VACV-Western Reserve (WR). Results of all analyses—parsimony, maximum likelihood, and Bayesian analysis (data not shown)—all generated 2 distinct Brazilian clades and indicated a close relationship between group 2 and VACV-WR. Neither group of BRZ-VACV was directly linked with VACV-Oswaldo Cruz Institute (IOC) or VACV-Lister in any analysis. Both BRZ-VACV groups are represented by 4 isolates each. VACV-Acambis3000 and VACV-modified vaccinia Ankara (MVA) group together with high support (98, Figure 2A), which reflects their derivation from vaccinia isolate Ankara (both are virus isolates of MVA). Isolates with known origins from Dryvax (Wyeth Laboratories, Marietta, PA, USA) (VACV-3737, -Acambis3, -Acambis2000, -DUKE) did not group together consistently. This could be a result of numerous passages, the history of Dryvax as a nonclonal vaccine strain, or both.

Genetic comparisons of the analyzed VACV strains can be seen in the online Technical Appendix. For BRZ-VACV at all 3 loci, the highest identity values occurred within

Table 1. Single gene sequences of *Vaccinia* viruses included in this study

Strain, isolate (abbreviation)	Gene and GenBank accession no.*			
	A56R	B19R	E3L	Rpo132-ATI-p4c-A27L
BeAn 58058 virus (BAV)	DQ206442	AF261890	DQ194388	NA
SpAn232 virus (SAV)	AF261890	DQ194384	DQ194387	NA
Belo Horizonte (VBH)	DQ206435	DQ194383	DQ194390	AF501620
Guarani P1 virus (GP1V)	DQ206436	DQ194380	DQ194385	DQ363383
Guarani P2 virus (GP2V)	DQ206437	DQ194381	DQ194386	NA
Araçatuba virus (ARAV)	AY523994	DQ194382	DQ194389	NA
Passatempo virus (PSTV)	DQ070848	DQ530239	DQ530240	NA
Cantagalo virus (CTGV)	AF229247	AY500815	AY771338	NA
Malbran virus (VACV-Malbran)	AY146624	NA	NA	NA
Vaccinia virus-Oswaldo Cruz Institute (VACV-IOC)	AF229248	AY500816	DQ070236	NA
BFL-3906	AF375077	NA	NA	NA
BFL-81	AF375078	NA	NA	NA
Wyeth	Z99051	Not included	NA	NA

*Reference: Vaccinia Copenhagen. A56R, viral hemagglutinin; B19R, soluble alpha/beta interferon [IFN] receptor; E3L, dsRNA-binding protein; rpo132-ATI-p4c-A27L, a region that codes for the major protein of the A-type inclusion body (*14*); NA, not available.

Table 2. Complete virus genome sequences of orthopoxviruses

Species	Strain, isolate (abbreviation)	GenBank accession no.
Vaccinia virus	3737 (VACV-3737)	DQ377945
	Acambis 3000 (VACV-Acambis 3000)	AY603355
	Acambis 2000 (VACV-Acambis 2000)	AY313847
	Acambis 3 (VACV-Acambis 3)	AY313848
	Vaccinia DUKE (VACV-DUKE)	DQ439815
	Lister (VACV-LIS)	AY678276
	Tian Tan (VACV-TianTan)	AY678275
	Western Reserve (VACV-WR)	AY243312
	Modified Vaccinia Ankara (VACV-MVA)	U94848
	Copenhagen (VACV-COP)	M35027
Horsepox virus	HPXV MNR-76 (HPXV)	DQ792504
Rabbitpox virus	Utrecht (RPXV-UTR)	AY484669
Cowpox virus	Brighton Red (CPXV-BR)	AF482758
	GRI-90 (CPXV-GRI)	X94355
Variola virus	Bangladesh-1975 (VARV-BSH 75)	L22579
	Garcia-1966 (VARV-GAR)	X76266
	India-1967 (VARV-IND)	X69198
Monkeypox virus	Congo_2003_ (MPX-RCG 2003)	DQ011154
	Liberia_1970_184 (MPXV-LIB 1970)	DQ011156
	USA_2003_039 (MPXV-USA 2003 039)	DQ011157
	WRAIR7-61 (MPXV-61 WR)	AY603973
	Zaire_1979-005 (MPXV-ZAI 1979)	DQ011155
	Zaire-96-I-16 (MPXV-ZAI 1996)	AF380138
Ectromelia virus	Moscow (ECTV-MOS)	AF012825
Camelpox virus	CMS (CMLV-CMS)	AY009089
	M-96 (CMLV-M96)	AF438165

each phylogenetic group. The E3L sequence of PSTV is the only exception. The PSTV E3L sequence is identical to those of GP1 and WR and showed the next highest identity level with the VBH sequence. This finding may indicate recombination in this portion of the genome between isolates from these 2 groups. Additional genomic data and analysis are needed.

The sequence diversity of the orthopoxvirus hemagglutinin (HA) gene has made it a potential marker for molecular diagnostics and phylogenetics (*24*). In particular, an 18-nt deletion within the gene has been proposed as an identifier of BRZ-VACV strains (*8*,*11*,*25*). A recent article describes 2 approaches for identifying BRZ-VACV–like isolates (*25*), both of which rely on presence of the 18-nt deletion. Figure 3 shows that while some Brazilian strains (ARAV, CTGV, GP2V, PSTV) share this deletion, others (SAV, BAV, GP1V and VBH) do not and would not be detected by these methods. Within the variably deleted region (Figure 3, amino acids 245–255), VACV isolates can be grouped into 4 types; Brazilian isolates fall into 2 of these. Rabbitpox virus from Utrecht has a unique deletion of only 2 residues (254, 255); VACV-3737 and VACV-Malbran share a 5–amino acid deletion (245–249). The VACV-3737 isolate was "plaque-purified from a vaccinia lesion following vaccination with Dryvax" (GenBank,accession no. DQ377945); VACV Malbran was used in vaccination programs in Argentina between 1937 and 1970 (*27*). GP1V and VBH share the same amino acid sequence (ADLY-DTYNDND; 245–255), identical to that of VACV-WR, VACV-Lister, and several other strains. The remaining isolates from Brazil share the familiar deletion of 6 amino acids (250–255) described previously. This deletion is also present in the VACV-IOC vaccine isolate (absent in Lister and VACV-WR). The variation in this region demonstrates that the deletion itself is not representative of all Brazilian isolates and therefore is not useful as an identifier of Brazilian VACV.

This same deletion has been recognized as a possible shared derived trait between VAVC-IOC and BRZ-VACV. Although this scenario is indeed possible for the CTGV-like BRZ-VACV of group 1, those isolates of group 2 are unlikely to be derived from the IOC vaccine strain because this strain does not carry the insertion. Furthermore, another variable gap occurs in this region of HA among isolates derived from VACV-Lister and Dryvax (New York City Board of Health [NYCBH]). Some clones present the deletion; others do not. The sequence can be found directly adjacent upstream of the variable deletion of the group 1 Brazilian isolates (Figure 3). Perhaps this region is nonessential and able to easily withstand such variation without detrimental effects on the utility of the HA protein or the fitness of the virus. Regardless, this deletion clearly cannot be relied upon to identify isolates directly related to Lister or NYCBH strains. A single shared deletion is only 1 char-

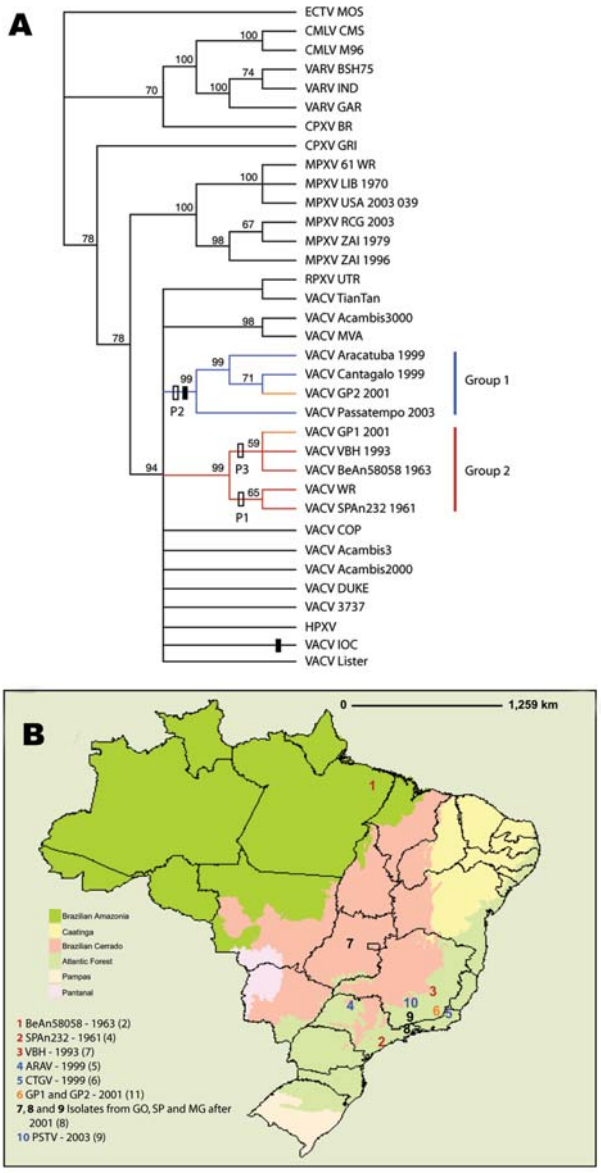

Figure 2. A) Strict consensus of the 6 most parsimonious trees derived from *Orthopoxvirus* species on the basis of B19R, E3L, and A56R sequences and rooted with ectromelia virus from Moscow (ECTV-MOS). Bootstrap values based on 100 bootstrap replicates of 10 random addition replicates each are shown at each node where the value was >50%. Black rectangles represent the 18-nt deletion shared by group 1 Brazilian vaccinia virus (BRZ-VACV) and VACV-Institute Oswaldo Cruz (IOC) and where it maps on the tree. Open rectangles represent the 3 digestion profiles (P1, P2, and P3) of the rpo132-ATI-p4c-A27L region assigned at each appropriate node. Blue, branches of group 1; red, branches of group 2; orange, isolation of VACV-Guarani P1 (GP1) and VACV-GP2 at adjacent farms in the same area. Colors correspond to information presented in panel B. B) Map of Brazil showing states and ecological biomes. Collection sites are represented by numbers that refer to Brazilian isolates and their respective years of isolation. Map used with permission from Instituto Brasileiro de Geografia e Estatistica. GO, Goiás; SP, São Paulo; MG, Minas Gerais. See Table 2 for other definitions.

acter toward determining phylogenetic relatedness among strains. If smallpox vaccines are not clones but rather pools of virions that vary at the molecular level, assuming relatedness based on a single molecular variation is even more difficult. Our phylogenetic analyses of 302 informative characters, including variation across the HA gene, does not indicate a close relationship between VACV-IOC and BRZ-VACV. The consensus tree (Figure 2A) depicts the IOC vaccine strain in an unresolved basal polytomy within the vaccinia cluster and is thus inconclusive as to the relatedness of VACV-IOC and BRZ-VACV.

Finally, published data indicate a large amount of variability in the region that includes the A-type inclusion body gene of the BRZ-VACV isolates (*5*,*9*,*11*–*13*). Figure 3B illustrates the arrangement of open reading frames in this region (rpo132-ATI-p4c-A27L) for CPXV Brighton Red, VACV Copenhagen, VACV-WR, and BRZ-VACV. The formation of A-type inclusion bodies is restricted to cells infected with CPXV, ectromelia virus, and raccoonpox virus. The C-terminus of the gene encoding the ATI protein is highly conserved among orthopoxviruses because it overlaps with the C-terminus of the RNA polymerase (132) coding region (*9*,*13*). However, the region between this conserved sequence and the N-terminus of the P4c precursor shows considerable variation among orthopoxvirus strains (*26*). Deletions and nucleotide changes generate variable coding regions and distinguishable digest patterns in this portion of the genome. Three profiles of this region have been discerned among BRZ-VACV; 2 are differentiated by distinct digestion patterns (*6*,*7*,*11*,*13*). The third state is the near complete absence of the A26L gene except for the last 112 nt (*9*,*13*). According to da Fonseca et al., SAV, a Brazilian VACV isolate, shows an A26L digestion profile identical to that of the VACV-WR strain (profile 1) (*6*). ARAV, PSTV, and GP2V show a digestion profile compatible with but not identical to that of the SAV, VACV-WR, and Lister strains (profile 2) (*7*,*11*,*13*). In BAV, VBH, and GP1V, a major portion of the A26L gene is missing (profile 3) (*5*,*9*,*13*). The open reading frame is small and is probably not expressed, although this has not been verified by Northern or Western blot. Damaso and colleagues demonstrated that A26L is present in CTGV; however, the digestion profile has not been established. Western blot analysis detected a 94-kDa protein typical of VACV-WR and VACV-IOC (*8*). These 3 profiles correspond to 3 clades recovered in the phylogenetic tree (Figure 2A). These findings constitute a major distinction between CTGV (also SAV, ARAV, PSTV, and GP2V, which exhibit profiles 1 and 2) and the other Brazilian isolates BAV, VBH, and GP1V (profile 3). Furthermore, the unusual deletion presented by BAV, VBH, and GP1V is remarkably different from the corresponding regions of VACV-IOC, -WR, and -Lister, strains implicated in the release of VACV in Brazil.

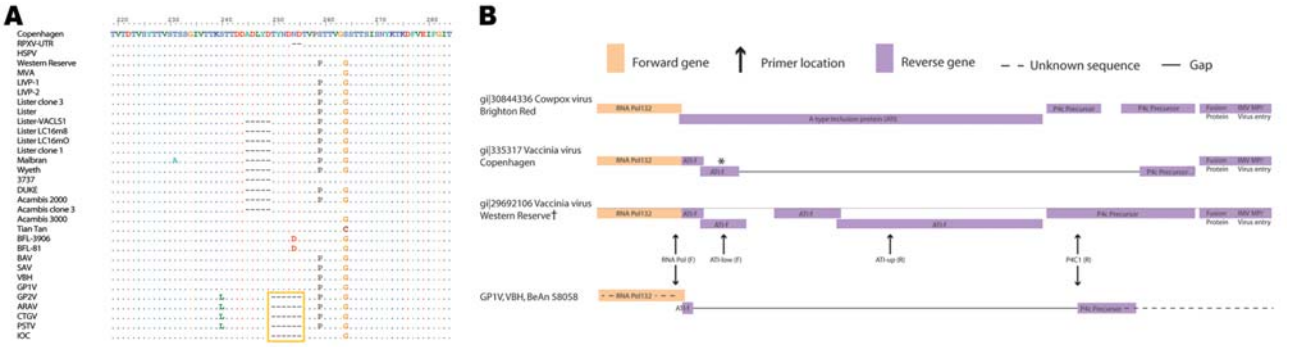

Figure 3. A) Partial amino acid alignment of A56R vaccinia virus Copenhagen (VACV-COP). Sequences were retrieved from GenBank and aligned using the BioEdit program (www.mbio.ncsu.edu/bioedit/bioedit.html). VACV-COP (M35027, 161908-161957) is used as the reference sequence; International Union of Pure and Applied Chemistry symbols, amino acids; dots, residues identical to the reference sequence; dashes, gaps created by the alignment; gold box surrounds the 18-nt deletion once proposed as a Brazilian (BRZ)-VACV molecular identifier. RPXV-UTR, rabbitpox virus-Utrecht; HSPV, horsepox virus; MVA, modified vaccinia Ankara; LIVP, Liverpool; BFL, buffalopox; BAV, BeAn 58058 virus; SAV, SpAn232 virus; VBH, Belo Horizonte virus; GP, Guarani; ARAV, Araçatuba virus; CTGV, Cantagalo virus; PSTV, Passatempo virus; IOC, Institute Oswaldo Cruz. B) Depiction of the rpo132-ATI-p4c-A27 region in cowpox virus Brighton Red (CPXV-BR) and VACV strains. Positions of primers RNApol, ATI-low, ATI-up, and P4c1 are illustrated. These primers have been used for the molecular diagnosis of Brazilian (BRZ)-VACV (*5,26*). The 300-bp fragment amplified by primers RNApol and P4c1 from GP1V and VBH was sequenced by Trindade et al. (*9,13*). Sequence outside this region is unknown. ATI-f is used to designate coding regions that resemble fragments of the wild-type (CPXV-BR) ATI gene. The gap in VACV-COP lies within the A26L gene. *This gene is often referred to as ATI due to its original description. However, the coding region is actually a fusion of the C-terminus of ATI and the N-terminus of P4c where the sequence in between has been deleted. The gene is currently identified by the Poxviridae Bioinformatics Resource (http://athena.bioc.uvic.ca/database.php?db=poxviridae) as belonging to the P4c precursor family. †Western Reserve is presumed similar in structure to SpAn232 virus, GP2V, ARAV, and PSTV due to similar restriction fragment length polymorphism results (*6,7,11,13*).

Discussion

Although eradication had been achieved in Europe by 1953 and in Central and North America by 1951, Brazil did not conduct a nationwide vaccination campaign during the 1950s. However, Brazilian people in cities and towns were vaccinated when outbreaks were reported by local authorities (*2*,*3*). Not until 1962 did Brazil, working with the Pan American Health Organization, launch a national campaign against smallpox (*3*). The intensified global WHO program prompted a renewed national program in Brazil in 1966. Fenner et al. (*3*) present a sample questionnaire that the smallpox eradication unit circulated in 1967 to all vaccine producers in countries accessible to WHO. In Brazil, this included 4 laboratories in different regions: the IOC, the Butantan Institute in São Paulo, the Institute for Biological Research in Porto Alegre, and an institute in the town of Recife, Pernambuco State (*3*). Records identify the seed strains used at these institutes to be "Paris," Lister, and "Lederle" (*28*). "Paris" appears to be a reference to the original animal vaccine imported from the Chambon Institute in 1887. The Institute for Biological Research in Porto Alegre received from London the Lister strain, which was originally developed at the Lister Institute, England. Recife and Butantan both indicated use of a strain obtained from Wyeth's Lederle Laboratory in the United States. The NYCBH strain was the source of Lederle and forerunner of Dryvax, the live vaccine maintained in the United States since the 1970s, and the research vaccine strain VACV-WR (*3*). These records confirm information that "Paris," Lister, and NYCBH were used in Brazil between 1968 and 1971 (*2*,*3*). It has been suggested that VACV-WR was used as a vaccine in Brazil as well; however, such use is now thought to be unlikely (*6*,*7*). The strain was derived by serial passage in mice infected intracranially and selected for neural virulence to mimic encephalitis, a rare side effect of the vaccine in humans (*29*,*30*). When available vaccine supplies dropped to critical levels in 1970, Brazil requested and received reserve vaccine from Argentina at least once (*3*). The Malbran strain was used in vaccination programs in Argentina until 1970, after which the Lister strain was adopted following WHO recommendations (*2*,*3*,*27*). Vaccine production at the Butantan Institute converted to the Lister strain late in 1970 (*3*), and Butantan became the official distributor for Brazil. Therefore, at least 4 strains of vaccine might have been distributed within Brazil during the WHO eradication campaign.

Discovery of Brazilian VACV Isolates

Since the 1960s, orthopoxviruses have been repeatedly isolated in Brazil and identified as VACV by classical immunologic, virologic, and molecular methods (*4*–*13*,*26*). In 1963, BAV was isolated from the blood of a rice rat (*Oryzomys* sp.*)* captured near the edge of a deforested area bordered by Amazon rain forest (Figure 2B) (*4*,*5*). This virus

is among the first orthopoxviruses naturally isolated from a wild rodent in Brazil (*4*,*5*). In the 1960s and 1970s, the Brazilian government and the Institute Adolfo Lutz conducted surveillance of arboviral activity in forested areas around the city of São Paulo, in the southeastern region of the country. During that investigation, a poxvirus was repeatedly isolated from sentinel mice and called Cotia virus (*31*). A sample of Cotia, SAV, was sent to the virus laboratory in Minas Gerais State. The specimen was plaque purified in duplicate, and a vaccinialike virus was isolated (*6*). Samples of Cotia have been studied elsewhere, and independent characterizations of the virus have been contradictory (*32*–*34*). These contradictory findings suggest that the material provided in the original sample(s) contained >1 virus, leading to conflicting reports of behavior and serologic relationships. We continue to refer to this sample as SPAn232 with reference to de Souza Lopes et al. (*31*) and da Fonseca et al. (*6*) to distinguish it from other isolates described previously.

In 1998, VBH was isolated from frozen clinical samples collected from mice during a mousepox-like outbreak in the animal facility of the Biological Institute of the University of Minas Gerais State in 1993 (Figure 2B) (*9*). Exanthematous outbreaks affecting dairy cattle and their handlers were reported in Brazil in 1999. Two new VACV strains, ARAV and CTGV, were isolated from sick cows in distinct geographic locations of the southeast region of the country (Figure 2C) (*7*,*8*). Since then, an increasing number of similar zoonotic outbreaks have been reported in different countryside areas of Brazil, particularly the southeast and southwest (Figure 2A, B) (*10*,*12*). In 2001, GP1V and GP2V were isolated from infected cows from adjacent farms near the area where CTGV had been found. Despite their coincident locale, the 2 new isolates constitute distinctly different VACV isolates (Figure 2A, C) (*13*). In 2003, PSTV was also isolated from cows in Minas Gerais State during a subsequent bovine vaccinia outbreak (Figure 2C; Table 3 [*11*]). Isolates from group 1, similar to CTGV, have been collected more often than those from group 2 (*10*,*25*), which could reflect greater fitness, prevalence, or virulence associated with group 1; however, any explanation is speculative. The difference might eventually be attributed to an as-yet unidentified sampling bias.

Isolates have been obtained from 3 of 6 Brazilian biome types (Figure 2B). Most have been collected in the Atlantic Forest region, where much of the forest has been cleared for dairy farms and coffee and sugar cane plantations (*35*). Reported human cases are typically in dairy workers, but SAV was isolated from a sentinel mouse placed within the Cotia Forest, São Paulo State (*6*), and BAV was obtained from a wild-caught rodent (*Oryzomys*) in northern Brazil. These occurrences, combined with the genetically similar isolates from the southern portion of the country (2,500 km away), indicate the potential circulation of viral strains throughout Brazil and perhaps other regions of South America. Moreover, some of these genetically divergent strains have a sympatric distribution, as illustrated by the isolation of GP1V (group 2) and GP2V (group 1) from adjacent farms (*13*). Sequencing of the more rapidly evolving inverted terminal repeats of the genome would help clarify the relationship between BRZ-VACV and its hosts. The near-terminal regions of the genome contain most genes involved in host interaction (*14*) and may illuminate ways in which the host may have shaped the virus evolution. No data are available regarding host variation, epidemiology, or evidence of clinical manifestations that can distinguish between the 2 genetic BRZ-VACV groups depicted in the phylogenetic analyses.

Potential Origins of Brazilian VACV

The possibility that the BRZ-VACV isolates originated from the spread of a smallpox vaccine strain, particularly

Table 3. Brazilian *Vaccinia* viruses and vaccine strains used in Brazil*

Virus	Year of isolation	Source	Place of isolation and biome	Reference
VACV-LIS	1870	Prussian soldier	Vaccine Institute, Cologne, Germany	(*3*)
VACV-WR	1876	NYCBH strain	New York City Department of Health Laboratory	(*3*)
SAV†	1961	Rodent, sentinel mice	São Paulo State, Atlantic tropical rainforest, Brazil	(*6*)
BAV	1963	Rodent, *Oryzomys sp.*	Para State, Amazon tropical rainforest, Brazil	(*4*,*5*)
VBH	1998	Rodent, BALB-c mice	Minas Gerais State, Cerrado woodland/savanna, Brazil	(*9*)
ARAV	1999	Cow	São Paulo State, Cerrado woodland/savanna Brazil	(*7*)
CTGV	1999	Cow	Rio de Janeiro State, Atlantic tropical rainforest, Brazil	(*8*)
GP1V	2001	Cow	Minas Gerais State, Atlantic tropical rainforest, Brazil	(*13*)
GP2V	2001	Cow	Minas Gerais State, Atlantic tropical rainforest	(*13*)
PSTV	2003	Cow	Minas Gerais State, Atlantic tropical rainforest, Brazil	(*11*)
VACV-IOC	ND†	Probable Paris strain	ND	(*3*,*8*)

*VACV, vaccinia virus; LIS, Lister; WR, Western Reserve; NYCBH, New York City Board of Health; SAV, SpAn232 virus; BAV, BeAn 58058 virus; VBH, Belo Horizonte virus; ARAV, Araçatuba virus; CTGV, Cantagalo virus; GP, Guarani virus; PSTV, Passatempo virus; IOC, Oswaldo Cruz Institute; ND, not determined.
†This virus was studied by the viral laboratory in Minas Gerais State in 1979 and cloned in Vero cells during the 1990s (*4*). It was originally collected and referred to as Cotia virus, which was repeatedly isolated throughout the 1960s and 1970s by the Adolfo Lutz Institute in Brazil (*33*).

VACV-IOC and VACV-Lister, has been proposed (*4*,*6*,*8*). A deletion in HA supports this hypothesis; but the larger portion of evidence, the phylogenetic analysis, does not. Available data offer no solid support for these proposed sources as the origin of BRZ-VACV. The VACV-IOC vaccine should be further examined to determine whether it contains virions without the 18-nt HA deletion. Historical records raise the possibility that strains currently circulating in Brazil were established from the original (first known) introduction of VACV to the region by the slave trade in the early part of the 19th century, by the introduction of the animal-cultivated vaccine in the latter part of the 19th century, or subsequently by the wave of VACV introduction brought through the smallpox eradication program in the mid 20th century. If BRZ-VACV was introduced from the Old World, multiple introductions from essentially the same source region (Europe) over ≈167 years (from at least 1804 to 1971, when vaccination in Brazil ceased) (*2*,*3*,*36*) could be responsible for the observed diversity of BRZ-VACV. Because the source of the naturally occurring isolates of Brazil may well be the same as that of the vaccine strains, the history of this viral species will be difficult to tease apart without direct sampling of naturally occurring isolates from Europe, particularly England, Germany, and France, where vaccine strains used worldwide are thought to have originated.

Vaccine escape has been hypothesized to account for other VACV isolated from domestic animals, including endemic buffalopox in India and HSPV (MNR-76) in Mongolia (*15*–*18*). A limited comparison between the Brazilian sequences and HSPV did not produce a monophyletic group, which makes it unlikely that they are derived from a very recent common ancestor such as one of the common vaccine strains developed in the past century. HSPV currently has the only complete genome available for comparison from a naturally occurring isolate. As the initial phylogenetic analysis of this sample suggests, we may find potential sources, such as group 1 of the BRZ-VACV, for vaccine strains circulating in various parts of the globe. Better sampling of naturally occurring VACV will be essential for determining the existence of indigenous VACV in Brazil.

The existence of 2 distinct groups of BRZ-VACV is clear. Their origins and how they are related remain undetermined. When more data become available, the 2 Brazilian groups may be found to be more closely related than illustrated by the tree in Figure 2. No epidemiologic data or clinical manifestations that differentiate isolates from the 2 groups have been reported. Epidemiologic data from recent and current zoonotic outbreaks could help elucidate what the genomic diversity implies. The data do establish a clear connection between group 2 (SAV, BAV, GP1V, and VBH) and NYCBH. VACV-WR is strictly a laboratory strain and has never been used as a vaccine (*29*,*30*). Complete genome sequencing of BRZ-VACV isolates and a sampling of clones from the original seed sample of NYCBH would be a dramatic step toward discerning the relationship between them. What differentiates the 2 lineages—history, ecology or recognizable phenotype—should be further investigated. The circulation of multiple variants across overlapping regions raises the possibility of recombination among variants, which may complicate the evolutionary history of BRZ-VACV.

The certification of global eradication of smallpox was an unprecedented event in human history. However, even now, the origins of VACV in nature and as a vaccine remain a mystery (*3*). During the smallpox eradication campaign, the dogma held that vaccine strains could not survive in nature and that wild-type vaccinia virus was extinct, yet VACV clearly persist today in Brazil and other parts of the world. If these isolates constitute a recently established zoonotic disease, they present a unique opportunity for understanding how molecular changes, such as recombination or other mutations, allow for the adaptation of poxviruses to new hosts and ecologic pressures.

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Dr Trindade is a virologist and a guest researcher at the Centers for Disease Control and Prevention in Atlanta, Georgia, USA. She has been actively involved in research projects examining the emergence of Vaccinia virus in Brazil. Her areas of interest include emergent viruses, molecular biology and viral diversity.

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Address for correspondence: Inger Damon, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G43, Atlanta, GA 30333, USA; email: idamon@cdc.gov

Large Water Management Projects and Schistosomiasis Control, Dongting Lake Region, China

Yue-Sheng Li,*† Giovanna Raso,†‡ Zheng-Yuan Zhao,*§ Yong-Kang He,* Magda K. Ellis,† and Donald P. McManus†

Construction of the Three Gorges Dam across the Yangtze River will substantially change the ecology of the Dongting Lake in southern China. In addition, the Chinese Central and Hunan Provinces' governmental authorities have instigated a Return Land to Lake Program that will extend the Dongting Lake surface area from the current 2,681 km^2 to 4,350 km^2.The previous construction of embankments and the large silt deposits made by the Yangtze River and other connecting rivers have contributed to frequent disastrous flooding. As a consequence of the 2 water projects, >2 million persons and their domestic animals are being resettled. This article provides an overview of the historical background of these 2 large water management projects, the associated population movement, and their impact on future transmission and control of schistosomiasis in the Dongting Lake area. The dam will likely substantially extend the range of the snail habitats and increase schistosome transmission and schistosomiasis cases.

Over a period of 2,200 years, until 1911, the Yangtze River has flooded 214 times, an average of 1 flood every 10 years. In the past century, 5 such floods have been severe. With the development of the Yangtze Basin, the economic cost of such flooding has also increased substantially. Controlling the flow of the Yangtze through the Three Gorges area would substantially reduce the danger of flooding in the lower plains regions and thus ameliorate economic losses. The construction of the giant Three Gorges Dam was first proposed in 1919, but construction did not begin until 1994. The entire Three Gorges project will be completed in 2009, and the dam's generated power is expected to supply ≈10% of the electricity needs of the People's Republic of China. After completion, the dam will be 2,300 m long and 185 m high, and the resulting 600-km-long reservoir created by the dam will inundate 115,000 acres of cultivated land. Hundreds of villages and countless historical relics and archeological and cultural sites will be submerged; ≈1.4 million persons will be resettled from this area (*1–3*). The construction of the dam will change the distribution of water and sand downstream from the dam and thus will have a strong effect on ecologic systems such as Dongting Lake in Hunan Province (Figure 1).

Dongting Lake is located at 28°30′–30°20′ N and 111°40′–113°40′ E in the northeastern part of Hunan Province and covers a water surface area of 2,681 km^2 (*3*). The heavily populated Dongting Lake basin is one of China's leading rice-producing regions; it is also known for its production of cotton and fish. Dongting Lake was China's largest lake during the Han dynasty. The rich sediment of the marshland attracted farmers, and several embankments

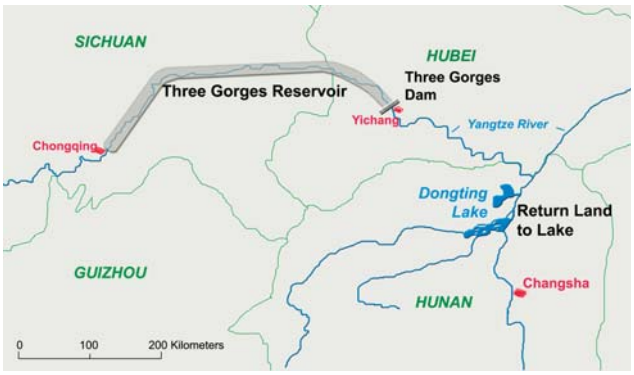

Figure 1. Location of the Three Gorges Dam and Reservoir across the Yangtze River and Return Land to Lake Program in the Dongting Lake region, Hunan Province, China.

*Hunan Institute of Parasitic Diseases, Yueyang, People's Republic of China; †Queensland Institute of Medical Research, Brisbane, Queensland, Australia; ‡University of Queensland, Brisbane, Queensland, Australia; and §Central South University, Changsha, People's Republic of China

were built to keep out the Yangtze River and to gain more farmland. Unfortunately, silting of mud and sand in the lake, in addition to the anthropogenic environmental transformations in the lowland areas, reduced the lake area and its storage capacity and caused rapid deterioration of the lake's flood diversion and flood storage functions. This diminishing capacity increased the occurrence of flood disasters, mainly because of the rupture of embankments. Until 1998, the lake had 228 embankments and was surrounded by a farmland area of 0.34 million ha. After a disastrous flood in 1998 (Figure 2A, B), which led to 3,656 deaths, made 378,000 persons homeless, and resulted in an economic loss of US $737 million, the State Council of the People's Republic of China formulated a policy, the Return Land to Lake Program, to prevent flooding. This program envisioned returning cultured lands into lake areas and included removing embankments and relocating residents from schistosomiasis-endemic areas to newly established towns. As a direct result, the Hunan government initiated a huge 4-year environmental project. The project involves moving 815,000 inhabitants of the Dongting Lake area inland and will result in the loss of >900,000 ha of farmland. Today, almost 90% of this project is completed (*4*,*5*).

As a consequence of these 2 huge water management projects, >2 million people and their domestic animals are being resettled. Large population movements are associated with extreme vulnerability to disease, especially due to malnutrition and lack of access to clean water and appropriate sanitation. Furthermore, officials are concerned that these water management projects will increase the transmission of schistosomiasis (*2*,*6*,*7*).

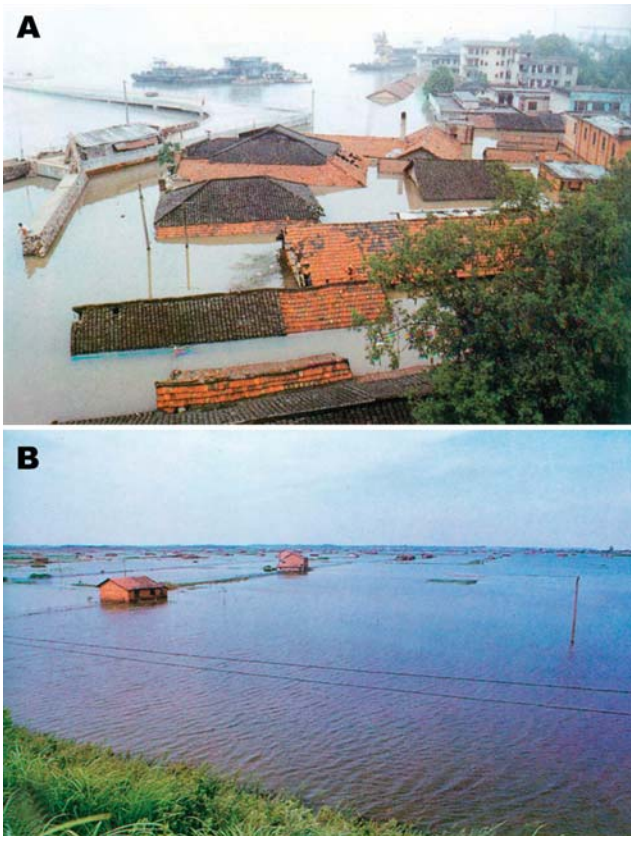

Figure 2. A) Submerged township houses in the Dongting Lake area due to the flood of 1998. B) An inundated rural area in 1998.

Schistosomiasis in Dongting Lake Area

Schistosomiasis japonica is a zoonotic parasitic disease caused by the trematode blood fluke *Schistosoma japonicum*, whose life cycle includes an amphibious freshwater snail, *Oncomelania hupensis hupensis*. The infection is transmitted by cercariae released by the snails and is contracted percutaneously by humans and other mammalian hosts, notably water buffaloes, when they are exposed to infested water. Schistosomiasis is a serious disease, which may become chronic, and remains a major health risk for the estimated >50 million persons living in the tropical and subtropical zones of China (7).

Archaeological studies have shown that schistosomiasis japonica has been endemic in the Dongting Lake region for thousands of years (*8*,*9*). In 1956, schistosomiasis was prevalent in 5 prefectures, 24 counties, and 14 state farms in the region; a total of 1 million persons and >300,000 domestic animals were infected with *S. japonicum,* and the total area of *Oncomelania* intermediate snail host habitats in the lake region was 3,795 km^2. After nearly half a century of control efforts, schistosomiasis is still endemic in 34 counties, and 6.12 million people are at risk for infection around Dongting Lake (Figure 3). Nearly 20% buffaloes were infected, and 200,000 active human cases were reported in 2003 (*10*). In the Dongting Lake region, schistosomiasis japonica particularly affects certain occupational groups, notably, farmers and fishermen, thus having substantial effects on the local economy and agricultural development of the area (*11*).

The continued concerted efforts to control schistosomiasis in China have reduced the initial peak estimates of human prevalences by >90%, and the national schistosomiasis control program for China is recognized as one of the most successful worldwide (*6*,*12*,*13*). Despite these remarkable achievements, schistosomiasis remains endemic in major foci in the marsh and lake regions of southern China, which cover a vast area of 5 provinces (Jiangsu, Anhui, Hubei, Jiangxi, and Hunan) and 2 mountainous regions (Sichuan and Yunnan) (*7*). Current estimates suggest that 843,007 humans and 74,000 bovines are infected (*7*,*14*). High-risk areas occur particularly around the Dongting and Poyang Lakes in the middle and lower reaches of the Yangtze River. Infected persons within these areas account for 86% of the total number of per-

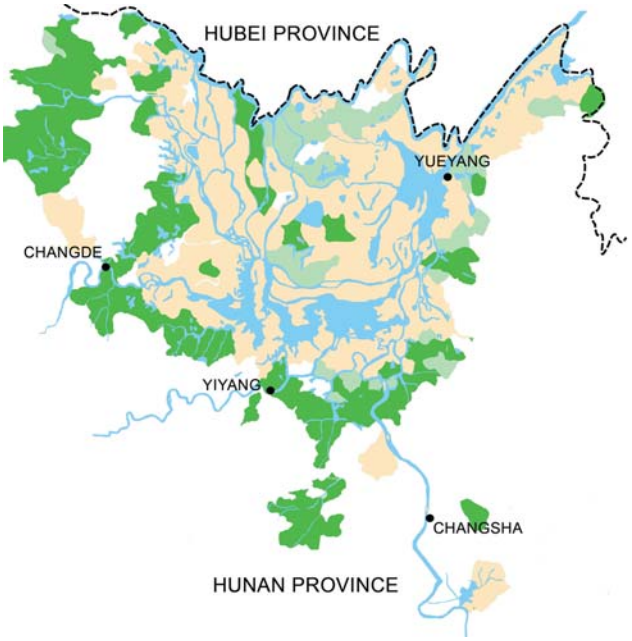

Figure 3. Hunan Province in 2002, showing that schistosomiasis is mainly confined to the area surrounding Dongting Lake. Areas in beige are classified as endemic for schistosomiasis. Areas in light green have fulfilled the transmission control criteria and are characterized by schistosomiasis infection rates <1% for humans and animals and a snail habitat reduction of >98%. Areas in dark green have fulfilled the criterion of interrupted transmission, which means that no new human or animal schistosomiasis cases occurred in successive years, and no snails were found for >1 year.

sons infected in the whole of China (*15*). Recent data from Sichuan Province suggest that schistosomiasis is reemerging in areas where the disease had been eliminated (*16*). With regard to the Dongting and Poyang Lakes, human reinfection with *S. japonicum* remains unacceptably high. Annually, up to 14% of patients who receive treatment are likely to become reinfected (*11*,*17*).

Water Management Projects and Schistosomiasis Transmission

As illustrated in a recent systematic review by Steinmann and colleagues, the development of water resources and their management can have increase schistosomiasis transmission (*18*). The meta-analysis, based on African studies, showed a risk ratio of 2.4 and 2.6 for urinary schistosomiasis (caused by *S. hematobium*) and intestinal schistosomiasis (caused by *S. mansoni*), respectively, among persons living adjacent to dam reservoirs. The analyses also showed that persons living near land that had been irrigated for agricultural use had an estimated risk ratio of 1.1 for urinary schistosomiasis and an estimated risk ratio of 4.7 for intestinal schistosomiasis. Furthermore, the same group estimated that 8.76 million Chinese persons live in irrigated, schistosome-endemic areas and that 9.97 million Chinese persons live in areas that are at high risk because of dam construction; however, they were unable to identify studies that assessed the effect of water resources development and management on schistosomiasis and called for additional screening of the Chinese literature (*18*).

To our knowledge, no published studies have yet actually measured the effect of the Three Gorges Dam on the transmission of schistosomiasis in the Dongting Lake region or in other water systems that connect with the 600-km-long reservoir resulting from the dam construction. However, several studies have used observed data on humans, animals, and snails in the region and knowledge gained from epidemiologic studies to predict the effect of ecologic changes on schistosomiasis transmission before and after construction of the dam. For example, sand and soil upstream of the dam are predicted to be deposited as silt in the Three Gorges reservoir (*17*,*19*), substantially reducing sand and soil downstream of the dam. Thus, one of the beneficial effects of the construction of the Three Gorges Dam would be an 85% reduction of yearly deposited sand and soil in Dongting Lake 50 years after closure**.** However, owing to less accumulation of silt deposits, a part of the marshland covered with reeds would degenerate into grass beaches or reed-grass beaches, which might increase snail breeding areas and potential for transmission of *S. japonicum*. In addition, the predictive models show that after 50 years, the sand and soil deposited in the Three Gorges reservoir would slowly discharge downstream, which might become a challenging problem for Dongting Lake in the future (*20*). Thus, the Three Gorges Dam will likely substantially extend the range of the snail habitats and increase schistosome transmission and the number of new schistosomiasis cases. Models inferred that when the dam is completed, the water level in Dongting Lake could increase by 0.06–1.5 m from January to May and decrease by 1.6–2.0 m from November to December. In the years soon after dam construction**,** the water level would not affect the distribution of snails. However, human and animal exposure to infested water can be expected to increase because the lake water would recede 3–7 days earlier than in previous years, and 17–25 days earlier than during drought periods (*20*). However, schistosomiasis surveillance and control cannot rely solely on such predictive models because the accuracy might be questionable; undertaking studies that measure the effect of the dam on *S. japonicum* transmission dynamics and schistosomiasis is thus of vital importance.

In some areas where snails were eliminated through the construction of embankments, snail habitats have reappeared with the implementation of the Return Land to Lake Program flood prevention policy, which includes the removal of embankments. In fact, the lake embankments not only prevent flooding and increase land for cultivation but also minimize snail habitats inside the embankment.

Preventing floods would automatically prevent *Oncomelania* snails from spreading throughout the lake region through breaking embankments. Such a spread of *Oncomelania* snails was documented in 5 villages when 1 year after the flood of 1996, antischistosomiasis workers visited a damaged embankment located in Huarong County, eastern Dongting Lake, and found that snails had spread to farmland areas (*21*) (Figure 4). The aforementioned concerns are confirmed by a study conducted in 41 villages distributed across different water systems of the Dongting Lake region (*5*). As shown in Figure 5, Cai and colleagues (*5*) showed that the Return Land to Lake Program had a negative effect on the *Oncomelania* snail distribution in 3 of the 6 investigated water systems. The authors estimated a >5-fold increase (from 0.2 km^2 to 1.8 km^2) of the snail areas due to the implementation of the program in the 6 investigated water systems of the Dongting Lake region (*5*). Overall, the area where the flood prevention policy is being implemented is as large as 6,670 km^2 and it is estimated that snails will recolonize ≈90% of the area from which they were eliminated in the past. The current snail habitat areas will likely double in the coming 3–5 years in the lake regions of China (*22*). Notably, marsh restoration is also under way in southern Iraq (*23*), with potential for substantial resurgence in *S. hematobium* transmission.

As a direct result of the Return Land to Lake Program, thousands of farmers and fishermen and their domestic animals are being resettled inland from the Dongting Lake coastal areas. Most of these migrants will be engaged in daily activities such as agriculture, fishing, and reed cutting and will unavoidably have contact with water infested with schistosome cercariae. A recent drug-based intervention study showed that water buffaloes are the major host reservoir for transmission of *S. japonicum* to humans in the lake region of China and account for ≈80% of transmission; the highest numbers of eggs were released into the environment by young buffaloes <1 year of age (*24*). The use of domesticated buffaloes as working animals by farmers will continue to provide reservoirs for *S. japonicum*. Schistosome infections due to occupational exposure may therefore reemerge in areas where schistosomiasis was previously reduced or eliminated, while compliance for diagnostic testing and treatment in Dongting Lake communities can be low (*25*,*26*). A recent longitudinal study that looked at schistosomiasis prevalences over 9 years (1997–2006) among mobile populations in Jicheng and Qingshanhu villages in the Dongting Lake region confirms these concerns (Figure 6). Jicheng and Qingshanhu are located in areas where the Return Land to Lake Program has been implemented; these 2 locations were occupied over time by migrants who pursued their economic activities, including fishing and animal farming. As a result, the prevalence of schistosomiasis increased steadily among the migrants

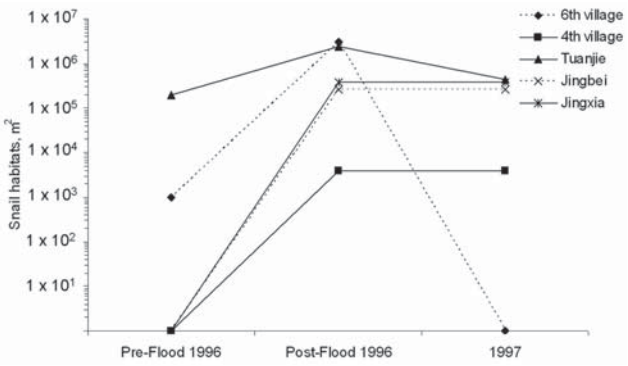

Figure 4. Influence of flooding in 1996 on the *Oncomelania* snail distribution in 5 villages (data from [*21*]).

and their animals. In Jicheng, persons remained engaged in fishing and animal farming during the whole study period, whereas in Qingshanhu, persons switched to industrial fish cultivation after 2001. Fish cultivation is a protective factor; thus, schistosomiasis prevalence decreased among these migrants, whereas in Jicheng, the prevalence of the disease continued to increase. Another study (Y.-K. He, unpub. data) on Dongting Lake showed that after persons were relocated from disease-endemic areas to 27 disease-endemic or -nonendemic villages, disease prevalence substantially increased in the former (Figure 7). Schistosomiasis prevalence decreased as the distance between the villages and Dongting Lake increased. Furthermore, schistosomiasis was again endemic in villages where it had previously been successfully eliminated. Another study showed that people who had been relocated from the Dongting Lake to hilly areas were at a decreased risk for infection with *S. japonicum* (*28*). On the other hand, migrants from disease-nonendemic areas, or areas where schistosomiasis was controlled, who are relocated to endemic areas will be at a high risk for infection because of limited natural immunity (i.e., persons never exposed to schistosomiasis) or diminished immunity (i.e., persons formerly exposed). Official

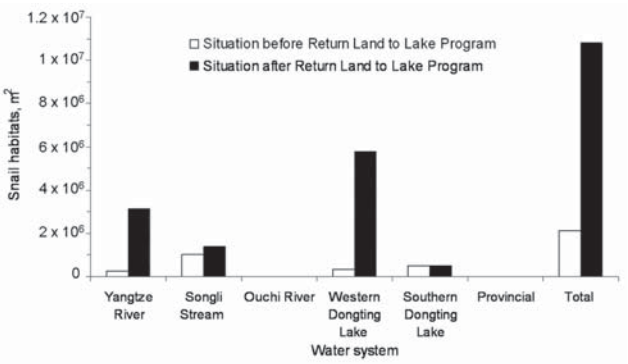

Figure 5. Influence of the Return Land to Lake Program on *Oncomelania* snail habitats in 6 different water systems in the Dongting Lake region (data from [*5*]).

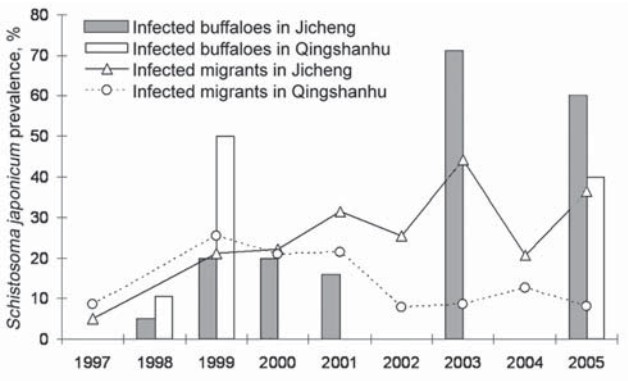

Figure 6. *Schistosoma japonicum* infection prevalences of migrants and water buffaloes in 2 areas in Dongting Lake over 9 years where the Return Land to Lake Program has been implemented (*27*). No bovine prevalence data were available for both villages for 1997, 2002, and 2004, and no human prevalence data were available for both villages for 1998. No buffaloes were present in Qingshanhu in 2000, 2001, and 2003.

statistics indicate that 5 new rural communities have been built around Dongting Lake for >2,000 migrants relocated from the schistosomiasis-nonendemic areas upstream of the Three Gorges Dam area at Chongqing (Figure 1). These migrants will likely be at high risk for *S. japonicum* infection if they contact infested water.

Future Control Strategies and Research Priorities

China's rapid economic development has resulted in the decentralization and market orientation of the health system (*29*,*30*). Unfortunately, treatment and prevention of schistosomiasis, which was receiving policy and programmatic advantages such as the provision of free medication and prevention, will undoubtedly be affected in terms of both efficiency and equity (*29*,*30*). Although the Chinese population has some awareness of schistosomiasis and its health effects, a considerable proportion of persons have no knowledge of preventive measures or are unwilling to pay for treatment (*31*). The health system currently in place may not satisfactorily meet the distinct needs of the changing population in the Dongting Lake area; the situation may be further exacerbated by the large population movements that are currently taking place. Studies are therefore needed that assess and quantify demographic, environmental, and socioeconomic changes on health outcomes in connection with the relocation of the 2 large migrant populations associated with the Three Gorges Dam project and the Return Land to Lake Program. Findings from these studies would enable the Chinese authorities to estimate and locate the actual population at risk and thus help with the strategic planning of future control efforts. Furthermore, health education will have to play a leading role in the control of schistosomiasis, especially in those communities with little or no prior knowledge of prevention and control or of drug treatment and other control strategies. School- and community-based health education that builds on persons'

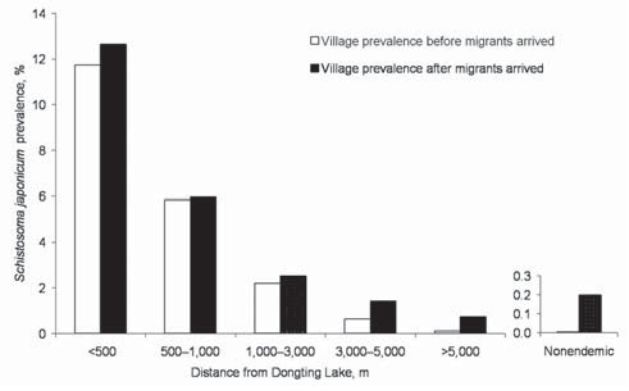

Figure 7. *Schistosoma japonicum* prevalences before and after migration due to the implementation of the Return Land to Lake Program in the Dongting Lake region.

Figure 8. A) Molluscicide with niclosamide for the control of *Oncomelania* snails in marshland between Dongting Lake and an embankment. B) Environmental modification to control *Oncomelania* breeding sites through canalization of water streams in Hunan Province.

preexisting knowledge and perceptions has a strong potential for raising knowledge and awareness of schistosomiasis (*32*,*33*).

In conclusion, integrated schistosomiasis control can be seen as a realistic target in the Dongting Lake region through strengthening of the schistosomiasis surveillance system already in place, which includes the monitoring of potential snail habitats, animal host reservoirs, and human cases. This monitoring is of central importance because with the implementation of the Return Land to Lake Program, local schistosomiasis endemicity might change (*4*). Current tools for controlling schistosomiasis may have to be combined or strengthened and strategies adapted according to social and environmental factors. Such strategies could include the following: improved access to treatment and preventive measures, health education, focal molluscicide (Figure 8A), environmental modification (Figure 8B), and improvement of sanitation and water-supply systems through the efforts of intersectorial collaboration and local economic development.

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Dr Li is director and professor at the Hunan Institute of Parasitic Diseases in Yueyang, China. His research interests include the epidemiology, diagnosis, and immunology of schistosomiasis and anti-schistosome vaccine development. He has been involved in schistosomiasis control in China for >30 years.

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Address for correspondence: Giovanna Raso, Public Health Building, School of Population Health, University of Queensland, Herston Rd, Herston, Queensland 4006, Australia; email: g.raso@uq.edu.au

Thottapalayam Virus, a Prototype Shrewborne Hantavirus

Jin-Won Song,* Luck Ju Baek,* Connie S. Schmaljohn,† and Richard Yanagihara‡

Thottapalayam virus (TPMV) has been placed in the genus *Hantavirus* of the family *Bunyaviridae* by virtue of its morphologic features and overall genetic similarities to well-characterized rodentborne hantaviruses. This virus has been isolated from the Asian house shrew (*Suncus murinus*); however, whether TPMV is naturally harbored by an insectivore host or represents spillover from a rodent reservoir host is unknown. Our analysis of published and unpublished data on the experimental host range, genetics, and molecular phylogeny of TPMV supports coevolution of TPMV with its nonrodent reservoir host. Future studies on the epizootiology of TPMV and investigations of new shrewborne hantaviruses will provide additional insights into the evolutionary origin of hantaviruses in their rodent and insectivore reservoir hosts. Such investigations may also provide clues about determinants of hantavirus pathogenicity and virulence.

Viruses in the genus *Hantavirus*, similar to other members of the family *Bunyaviridae*, have a negative-sense, single-stranded RNA genome in 3 segments designated large (L), medium (M), and small (S), which encode an RNA-dependent RNA polymerase, envelope glycoproteins (Gn, Gc) and nucleocapsid (N) protein, respectively (*1*,*2*). Each viral genomic segment has the identical 3′-terminal sequence of AUCAUCAUCUG, which is unique to hantaviruses (*3*). However, unlike the >200 other members in this virus family, most of which have arthropod vectors, each genetically distinct hantavirus is harbored by 1 or a few closely related rodent species with which it appears to have coevolved (*4*,*5*). Hantaan virus (HTNV) shares a multimillennium relationship with the striped field mouse (*Apodemus agrarius*), Dobrava virus (DOBV) with the yellow-necked field mouse (*Apodemus flavicollis*), Seoul virus (SEOV) with the Norway rat (*Rattus norvegicus*), Thailand virus (THAIV) with the bandicoot rat (*Bandicota indica*), Puumala virus (PUUV) with the bank vole (*Myodes glareolus*, formerly *Clethrionomys glareolus*), Tula virus (TULV) with the European common vole (*Microtus arvalis*), Prospect Hill virus (PHV) with the meadow vole (*M. pennsylvanicus*), and Sin Nombre virus (SNV) with the deer mouse (*Peromyscus maniculatus*).

Many other rodent-hantavirus associations are known, including the recent discovery of a hantavirus in the African wood mouse (*Hylomyscus simus*) (*6*). Until recently, the 1 exception that did not have a confirmed rodent association has been Thottapalayam virus (TPMV), which was isolated from an Asian house shrew or musk shrew (*Suncus murinus*) captured in 1964 during a survey for Japanese encephalitis virus in southern India (*7*). TPMV has been classified as a hantavirus by virtue of its ultrastructural features (*8*) and overall genetic similarities with well-characterized rodentborne hantaviruses (*9*,*10*). Although isolation of TPMV predates that of all other hantaviruses, including prototype HTNV, little is known about its biology and genetics. Whether TPMV is naturally harbored by the Asian house shrew or represents recent spillover from a rodent reservoir host is unknown. We present previously unpublished data on experimental TPMV infection in small laboratory animals. We also summarize information on the antigenic and phylogenetic relationships between TPMV and rodentborne hantaviruses that may cause hemorrhagic fever with renal syndrome (HFRS) (*11*) or hantavirus pulmonary syndrome (HPS) (*12*).

TPMV Infection in Cell Culture

Shortly after TPMV was isolated, in vitro studies involving primary cultures of guinea pig embryonic kidney, lung, and heart cells supported replication of this virus (*13*). Although mild cytopathic effect was observed in these cell

*Korea University College of Medicine, Seoul, South Korea; †US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA; and ‡University of Hawaii at Manoa, Honolulu, Hawaii, USA

cultures, the kinetics of TPMV replication was not vigorously studied. Recent adaptation of the VRC-66412 strain of TPMV to the E6 clone of Vero cells (CRL 1586) showed no cytopathic effect. At a multiplicity of infection of 0.1, intracytoplasmic, virus-specific granular fluorescence appeared somewhat later in Vero E6 cells infected with TPMV than in those cells infected with HTNV or PUUV. Strains of HTNV isolated from striped field mice and strains of SEOV from Norway rats produce large plaques (6 mm diameter) on Vero-E6 cell monolayers stained 6 days after infection with neutral red. In contrast, the VRC-66412 strain of TPMV produces much smaller plaques (≈1–1.5 mm diameter) by staining with neutral red. These plaques are easily enumerated by immunohistochemical staining 12 days after infection.

Experimental TPMV Infection in Laboratory Animals

In their primary rodent reservoir hosts, naturally occurring and experimentally induced hantavirus infections are subclinical and chronic (*14–20*). Experimental infection of striped field mice and bank voles with HTNV and PUUV, respectively, is characterized by transient viremia and short-lived shedding of virus in oropharyngeal secretions; prolonged excretion of virus in urine, feces, or both; and virus persistence in tissues, particularly lung (*14*,*15*,*17–20*). PUUV has been serially passaged only in laboratory-bred bank voles (*17*,*19*,*20*), and strains Hällnäs and K27 of PUUV cause an asymptomatic persistent infection in Mongolian gerbils (*21*) and Syrian hamsters (*22*), respectively. Horizontal intracage transmission has been demonstrated for HTNV and PUUV, but vertical transmission does not appear to occur (*15*,*17*,*20*,*23*). In infant mice and rats experimentally infected with HTNV and SEOV, respectively, fatal meningoencephalitis develops (*24–27*). In contrast, mice and rats >14–21 days of age are generally resistant to experimental HTNV and SEOV infection (*26*,*27*). Conversely, infant mice are resistant to experimental infection with PUUV (*18*,*19*), PHV (L.J. Baek, unpub. data), and SNV, the prototype sigmodontine rodentborne hantavirus that causes HPS (*28*).

To determine the host range of experimental TPMV infection and to ascertain whether susceptibility of small laboratory animals to disseminated TPMV infection is age-dependent, we infected NIH Swiss mice and Mongolian gerbils of different ages, as well as infant deer mice and gray short-tailed opossums (*Monodelphis domestica*), by the intracerebral route with 6,000 PFU of TPMV (Table 1). Infant Swiss NIH mice, deer mice, and gerbils were equally susceptible to fatal TPMV infection. Moreover, susceptibility to disseminated TPMV infection in NIH Swiss mice and gerbils was not age-dependent, as shown by lethal meningoencephalitis (characterized by hyperexcitability, ataxia, limb paralysis, and seizures) in animals infected at 1–21 days of age. TPMV antigen was detected in cryostat-cut sections of lung, brain, kidney, spleen, and liver of experimentally infected, moribund NIH Swiss mice and gerbils (Figure 1). Thus, unlike HTNV, SEOV, PUUV, PHV, and SNV, TPMV appears to have a much broader experimental host range in small laboratory animals.

Studies now in progress on experimental TPMV infection in laboratory-reared Asian house shrews should provide information about virus carriage and shedding. In addition, experimental demonstration of subclinical and chronic TPMV infection in Asian house shrews would also support the nonrodent reservoir host status.

Antigenic, Genetic, and Phylogenetic Characterization

The antigenic relationship of TPMV with 31 other hantavirus isolates has been investigated by ELISA and cross plaque-reduction neutralization tests (PRNTs) by using antisera from experimentally infected animals (*9*). Antisera prepared against strains of HTNV, SEOV, THAIV, PUUV, and PHV have 16-fold or lower ELISA titers to cell culture-derived TPMV antigen than to homologous antigen (*9*). ELISAs with monoclonal antibodies (MAbs) prepared against HTNV showed that certain epitopes defined by Gc-

Table 1. Susceptibility of small laboratory animals of various ages to fatal Thottapalayam virus meningoencephalitis*

Host species	Age at injection, d	Illness onset, d	Mortality rate, %
Mus musculus	1	7	100
	5	7	100
	10	6	88
	14	6	94
	21	8	67
Meriones unguiculatus	1	9	100
	5	11	100
	11	7	80
	16	14	100
Peromyscus maniculatus	4	11	100
Monodelphis domestica	30	–	0

*Animals of various ages were injected intracerebrally with 6,000 PFU of Thottapalayam virus strain VRC-66412 and examined daily for neurologic signs and death.

specific MAbs, but not Gn-specific MAbs, are conserved among most hantaviruses, including TPMV (*9*,*29*). Similarly, cross-immunoprecipitation of radionuclide-labeled TPMV and HTNV proteins have shown conserved N and Gc glycoprotein epitopes but not Gn epitopes (*9*). Of the 32 hantaviruses examined by PRNT, TPMV is the only virus that shows no cross-neutralization with any other hantavirus, i.e., none of the heterologous antisera neutralizes TPMV, and the antiserum to TPMV does not neutralize any other hantavirus.

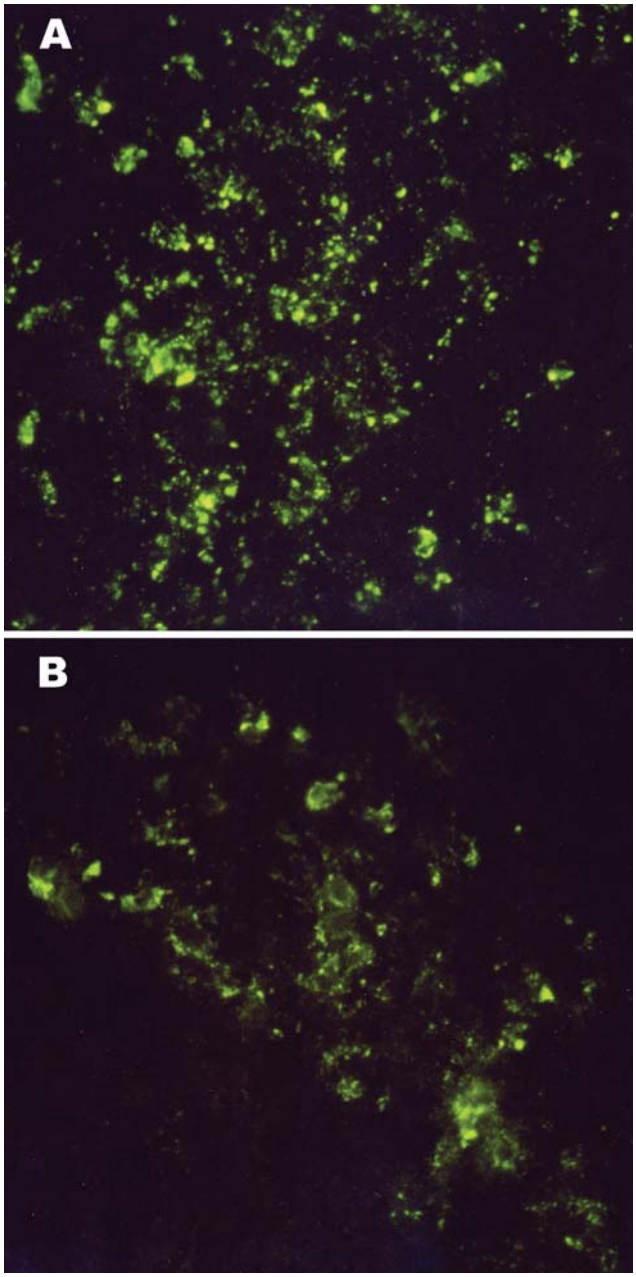

Figure 1. Intracytoplasmic virus-specific fluorescence in brain tissues of an 11-day-old Mongolian gerbil (A) and a 10-day-old NIH Swiss mouse (B) injected intracerebrally with 6,000 PFU of Thottapalayam virus (TPMV) strain VRC-66412 from serum of an adult rat injected intramuscularly with TPMV (original magnification, x400).

Apart from being antigenically distinct, TPMV also appears to be the most genetically divergent member of the *Hantavirus* genus. Full-length S-segment nucleotide and deduced amino acid sequences have nearly the same calculated distances from all other hantaviruses, which suggests an early evolutionary divergence (Table 2). Phylogenetic analysis based on the N protein–encoding S segment, as determined by the maximum parsimony and neighbor-joining methods, supports this conclusion, in that TPMV is an outgroup and all other hantaviruses segregate into clades, which parallel the evolution of murid, arvicolid, and sigmodontine rodents (Figure 2). Further elucidation of the molecular phylogeny of TPMV has been hampered by the lack of TPMV M- and L-segment sequence information. After many failed attempts, the M- and L-genomic segments of TPMV have recently been fully sequenced (J.-W. Song, R. Yanagihara, unpub. data). Full-genome analysis of TPMV shows phylogenetic relationships with rodentborne hantaviruses, which are congruent with those formed only on the basis of the S segment.

TPMV as a Human Pathogen

Hantaviruses possess strikingly different degrees of pathogenicity for humans. Many viruses, particularly those harbored by arvicolid rodents, appear to be avirulent (such as PHV) or have low pathogenic potential (such as TULV). Among the HFRS- and HPS-causing pathogenic hantaviruses, differential use of β-3 integrins as cellular receptors on platelets and endothelial cells may account for vascular leakage and hemorrhage associated with HFRS and HPS (*30*,*31*). Preliminary studies indicate that TPMV, like PHV, uses β-1 rather than β-3 integrin (I.N. Gavrilovskaya, R. Yanagihara, unpub. data), which suggests that TPMV is nonpathogenic.

Immmunoglobulin G (IgG) against HTNV has been detected in sera from persons in southern India (*32*,*33*), but evidence for hantavirus disease in India is lacking. Nevertheless, because several species of *Apodemus* mice, including the wood mouse (*A. sylvaticus*), are present in India, the demonstrated seroreactivity to HTNV may represent cross-reactivity with another *Apodemus*-borne hantavirus. Alternatively, seroreactivity may indicate infection with a nonrodentborne hantavirus, such as TPMV. Because Asian house shrews are peridomestic, frequently living within or in close proximity to human dwellings, TPMV infection may occur in humans.

To begin to address this issue, researchers collected serum specimens from 363 life-long residents of Mumbai, India, during 1992 and 1993 as part of a study of retroviral infections, and tested them for serologic evidence of TPMV and SEOV infection by using the indirect immunofluores-

Table 2. Comparison of full-length small-segment nucleotide and amino acid sequences of hantaviruses with Thottapalayam virus*

	Thottapalayam virus	
Hantavirus (strain)	1,530 nt	436 aa
Hantaan (76–118)	47.9	47.1
Seoul (HR80–39)	40.8	45.7
Puumala (Sotkamo)	43.7	44.6
Prospect Hill (PH-1)	47.5	44.3
Sin Nombre (NMH10)	41.2	47.9
Andes (Chile 9717869)	44.2	47.2

*Values are percentage similarities. Distances were calculated using PAUP version 3.1.1 (Sinauer Associates Inc., Sunderland, MA, USA).

cent antibody technique. A total of 12 (3.3%) serum samples were reactive to TPMV (geometric mean titer 80.6), and 16 (4.4%) samples were reactive to SEOV (geometric mean titer 103.1). Attempts to verify the specificity of this immunoreactivity by PRNT were unsuccessful (J.-W. Song, unpub. data). More recently, however, evidence suggestive of TPMV infection was found in a Laotian immigrant with a febrile illness by using a Western immunoblot analysis and a newly developed ELISA. This ELISA used a recombinant TPMV N antigen that contained an E5/G6 epitope captured by MAb E5/G6 (*29*).

Future Research Directions and Perspective

Although the detection of viruses in insectivores has been largely incidental or accidental, demonstration of Borna disease virus in brain tissues of the bicolored white-toothed shrew (*Crocidura leucodon*) (*34*) suggests that insectivores may play a greater role in the ecology of zoonotic diseases than previously appreciated. The prototype shrewborne hantavirus, TPMV, must be viewed within this context. Although limited data do not indicate that TPMV is a human pathogen, other shrewborne hantaviruses may be pathogenic for humans. In this regard, no one had the prescience to predict that hantaviruses harbored by sigmodontine rodents would be etiologically associated with an acute, rapidly progressive, frequently fatal respiratory illness in the Americas, now known as HPS. The realization that rodentborne hantaviruses are capable of causing diseases as clinically disparate as HFRS and HPS increases the possibility that hantaviruses harbored by nonrodent hosts may similarly cause a wide spectrum of febrile diseases or be linked with a syndrome currently of unknown etiology. Development of reagents directed toward insectivore serum proteins would greatly increase the sensitivity and specificity of serologic assays to ascertain antihantaviral immunologic responses in shrews and result in improved screening for new shrewborne hantaviruses.

Even in the absence of such reagents, several insectivore species are already known to be prime candidates for intensive investigations aimed at identifying new hantaviruses and exploring their disease associations. As examples, hantavirus antigens have been previously detected in tissues of the Eurasian common shrew (*Sorex araneus*), Eurasian water shrew (*Neomys fodiens*), and common mole (*Talpa europea*) in the former Soviet Union (*35*,*36*), and seroreactivity suggestive of hantavirus infection was found in short-tailed shrew (*Blarina brevicauda*) in the United States (*37*). In addition, hantaviruses isolated more than 2 decades ago from the greater white-toothed shrew (*Crocidura russula*) (*38*) and Chinese mole shrew (*Anourosorex squamipes*) (*38*) in Sichuan Province, People's Republic of China, have been inadequately characterized. The probability is high that >1 of these shrewborne hantaviruses may be phylogenetically distinct.

Another approach to the targeted discovery of new hantaviruses harbored by shrews relies on molecular phylogeny. By constructing phylogenetic trees based on mitochondrial or nuclear gene DNA sequences, existence of hantaviruses in the Korean field mouse (*A. peninsulae*) (*39*) and the royal vole (*Myodes regulus,* formerly *Eothenomys regulus*) (J.-W. Song, unpub. data) was correctly predicted. When this predictive paradigm is applied to insectivores,

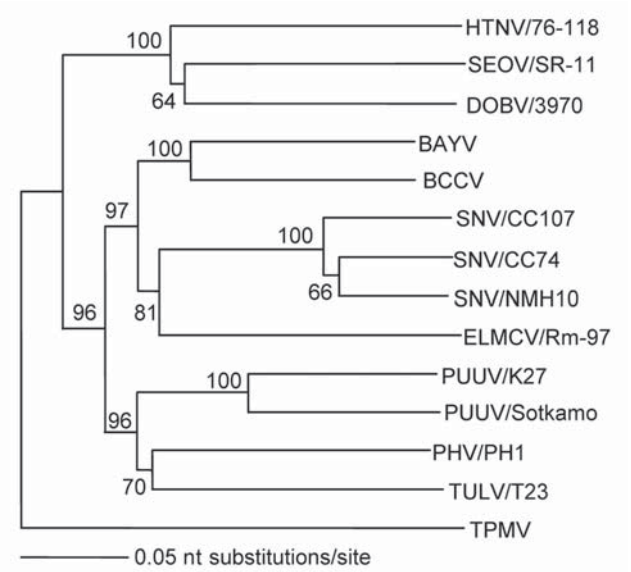

Figure 2. Phylogenetic relationship between Thottapalayam virus (TPMV) and other hantaviruses based on the nucleotide sequences of the full-length small (S) genomic segment, determined by using the neighbor-joining method. Numbers at each node are bootstrap probabilities (expressed as percentages) determined for 1,000 iterations. Branch lengths are proportional to number of nucleotide substitutions per site. Sequences used for comparison were those of Hantaan (HTNV/76–118, NC 005218), Seoul (SEOV/SR-11, M34881), Dobrava (DOBV/3970, L41916), Bayou (BAYV, L36929), Black Creek Canal (BCCV, L39949), Sin Nombre (SNV/CC107, L33683; SNV/CC74, L33816; and SNV/NMH10, L25784), El Moro Canyon (ELMCV/Rm-97, U11427), Puumala (PUUV/K27, L08804 and PUUV/Sotkamo, NC 005224), Prospect Hill (PHV/PH1, Z49098), and Tula (TULV/T23, Z30945) viruses. Strain designations are unavailable for BAYV and BCCV. The full-length S-segment sequence of TPMV has been deposited into GenBank (accession no. AY526097).

species of *Crocidura* and *Sorex* genera would be expected to serve as reservoir hosts of hantaviruses because of their close phylogenetic proximity to *S. murinus* (Figure 3). The recent detection of a novel hantavirus in the Therese shrew (*Crocidura theresae*) in Guinea (*40*) supports this conjecture. In addition, aided by primers based on the complete genome of TPMV, new hantaviruses have been found in 4 shrew species in the family *Soricidae* from Eurasia and the Americas (J.-W. Song, R. Yanagihara, unpub. data, and S. Arai, R. Yanagihara, unpub. data). These newly identified shrewborne hantaviruses provide new knowledge about the genetic diversity of hantaviruses as well as possible insights into their evolutionary origin through host-switching events.

Fundamental to the discovery and characterization of new hantaviruses, whether harbored by insectivores or rodents, is their relevance to human health. Because insectivore populations are generally much smaller than rodent populations, the probability of contact between humans and most insectivore species (and their excretions) may be too low for virus transmission. However, this probability is true for most zoonotic microbes, which only rarely infect humans. Thus, in the absence of disease outbreaks, zoonotic diseases frequently go unrecognized. In this regard, HPS would have similarly gone undetected had cases not clustered in time and space and had a closely knit group of dedicated and astute healthcare workers not recognized that something unusual was happening. The long-awaited clue of finding IgG against TPMV in a febrile Laotian immigrant (*29*) might indicate cross-reactivity to a pathogenic shrewborne hantavirus in Southeast Asia.

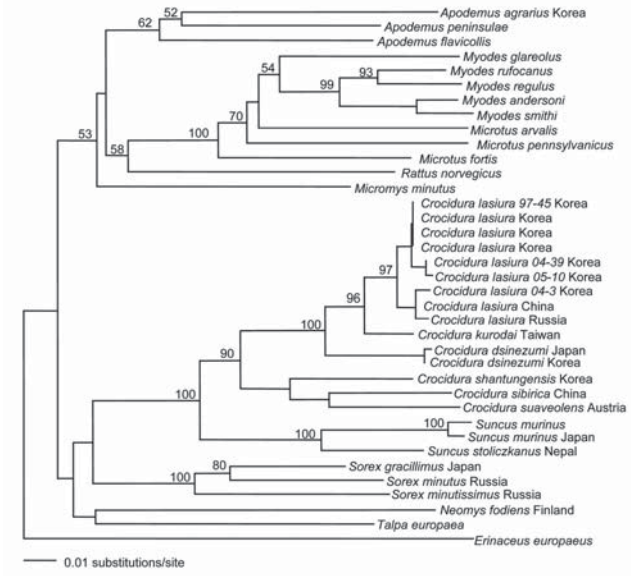

Figure 3. Phylogenetic relationship between *Suncus murinus* and other insectivores and rodents in a 401-nt cytochrome b region of mitochondrial DNA determined by using the neighbor-joining method. Numbers at each node are bootstrap probabilities determined for 1,000 iterations. Members of the genus *Crocidura* (white-toothed shrews) belong to the subfamily *Crocidurinae*. They are distinguished from members of the subfamily *Soricinae* (red-toothed shrews) by their unpigmented teeth, 3 upper unicuspids, and more prominent ears than either the genera *Sorex* or *Neomys*.

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Dr Song is professor of microbiology at Korea University. His research interests include global epizootiology and epidemiology of hantaviruses.

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Address for correspondence: Richard Yanagihara, Pacific Center for Emerging Infectious Diseases Research, John A. Burns School of Medicine, University of Hawaii at Manoa, 651 Ilalo St, BSB 320L, Honolulu, HI 96813, USA; email: yanagiha@pbrc.hawaii.edu

Virulence Characteristics of *Klebsiella* and Clinical Manifestations of *K. pneumoniae* Bloodstream Infections

Victor L. Yu,* Dennis S. Hansen, † Wen Chien Ko,‡ Asia Sagnimeni,* Keith P. Klugman,§ Anne von Gottberg,¶ Herman Goossens,# Marilyn M. Wagener,* Vicente J. Benedi,[1] and the International Klebsiella Study Group[2]**

We studied 455 consecutive episodes of *Klebsiella pneumoniae* bacteremia occurring in 7 countries. Community-acquired pneumonia and an invasive syndrome of liver abscess, meningitis, or endophthalmitis occurred only in Taiwan and South Africa. Infections by K1 and K2 capsular serotype, the mucoid phenotype, and aerobactin production were important determinants of virulence. The mucoid phenotype was seen in 94% of isolates in patients with community-acquired pneumonia and in 100% of isolates that caused the invasive syndrome in Taiwan and South Africa, compared with only 2% of isolates elsewhere. Mortality of mice injected with mucoid strains (69%) was strikingly higher than that occurring in mice injected with nonmucoid strains (3%, p<0.001). Differences in clinical features of bacteremic infection with *K. pneumoniae* are due to the virulence factors expressed by the organism.

In the past decade, geographic differences have been recognized in the spectrum of disease caused by *Klebsiella pneumoniae*. These differences include a preponderance of severe invasive disease in Taiwan and other parts of Asia (*1*–*8*). A characteristic syndrome has emerged in which liver abscess is accompanied by *K. pneumoniae* bacteremia and sometimes by endophthalmitis or meningitis. This is typically a community-acquired infection that occurs in patients with diabetes mellitus. Reports of this syndrome from North America, Europe, and Australia are uncommon (*2*).

Additionally, *K. pneumoniae* has long been recognized as a possible cause of community-acquired pneumonia. Over the past 2 decades, *K. pneumoniae* has been an exceedingly rare cause of community-acquired pneumonia in North America, Europe, and Australia (*2*,*9*,*10*). Yet, it remains an important cause of severe community-acquired pneumonia in Asia and Africa (*11*–*15*). In these regions, patients also have classic risk factor of alcoholism (*2*).

We have completed a prospective study of 455 patients from 7 countries with *K. pneumoniae* bacteremia (*2*). We found that although nosocomial infections with *K. pneumoniae* occurred worldwide, some manifestations of community-acquired infection (namely, liver abscess and community-acquired pneumonia) were geographically restricted. These manifestations of disease occurred almost exclusively in Taiwan and South Africa (*2*). Potential explanations for these geographic differences in clinical manifestations include host factors such as rates of diabetes mellitus, alcoholism, access to healthcare, and socioeconomic factors.

Another explanation for these differences is related to the organism. In this study, we performed capsular serotyping, determined the presence of mucoid phenotype and aerobactin production, and assessed lethality in a murine model and correlated these in vitro and in vivo results with

*University of Pittsburgh, Pittsburgh, Pennsylvania, USA; †Statens Serum Institut, Copenhagen, Denmark; ‡National Cheng Kung University Medical College, Tainan, Taiwan, Republic of China; §Emory University, Atlanta, Georgia, USA; ¶South African Institute of Medical Research, Johannesburg, South Africa; #University Hospital, Antwerp, Belgium; and **Universidad de las Islas Baleares, Palma de Mallorca, Spain

[1]Deceased.

[2]The International Klebsiella Study Group comprises the previously named authors plus Jose Maria Casellas, Gordon Trenholme, Joseph McCormack, Sunita Mohapatra, and Lutfiye Mulazimoglu.

the clinical manifestation of patients with *K. pneumoniae* bloodstream infections. Our aim was to determine whether the different manifestations of infection occurring in different geographic regions could be correlated with differences in organism characteristics.

Methods

Study Design

A prospective, observational study of consecutive, sequentially encountered patients with *K. pneumoniae* bacteremia was conducted in 12 hospitals in the United States, Taiwan, Australia, South Africa, Turkey, Belgium, and Argentina. No patients were excluded from analysis. The study period was January 1, 1996, to December 31, 1997. Patients >16 years of age with positive blood cultures for *K. pneumoniae* were enrolled and completed a 188-item study form. Patients were followed up for 1 month after the onset of bacteremia to assess clinical outcome, including deaths and infectious complications. The study was observational in that administration of antimicrobial agents and other therapeutic management were controlled by the patient's physician, not the investigators. The study was approved by institutional review boards as required by local hospital policy.

Definitions

Terms were defined a priori (that is, before data analysis). Community-acquired bacteremia was defined as a positive blood culture taken on admission or within 48 hours of admission. Site of infection accompanying the bacteremia was determined as pneumonia, urinary tract infection, meningitis, incisional wound infection, other soft tissue infection, intraabdominal infection, and primary bloodstream infection by using Centers for Disease Control and Prevention definitions (*16*). "Invasive" infections accompanying *K. pneumoniae* bacteremia were further defined as liver abscess, meningitis, or endophthalmitis. Liver abscess was defined by the coexistence of blood cultures positive for *K. pneumoniae* and evidence of an intrahepatic abscess cavity by ultrasonography or computed tomography. Meningitis was defined as culture of *K. pneumoniae* from the cerebrospinal fluid. Endophthalmitis was defined as decreased visual acuity, pain, hypopyon, or severe anterior uveitis concurrent with *K. pneumoniae* bacteremia in a patient.

Microbiology

Blood culture isolates of *K. pneumoniae* were sent by the participating hospitals on nutrient agar slants to the Special Pathogens Laboratory in Pittsburgh. There, the identity of each isolate as *K. pneumoniae* was confirmed by using the Vitek GNI system (bioMérieux Vitek, Hazelwood, MO, USA).

The isolates were classified phenotypically as mucoid or nonmucoid. Colonies were touched with a loop; the loop was then lifted vertically from the surface of the agar plate. Mucoid phenotype was defined as being present when a stringlike growth was observed to attach to the loop as it was lifted from the plate (Figure 1). Presence of the *rmpA* gene (rmp = regulator of the mucoid phenotype) was sought by DNA dot blot hybridization by using a 640-bp probe (position 478–1117 of the *rmpA* gene; accession no. X17518). The probe was produced by direct digoxigenin (DIG)-labeled PCR by using the PCR DIG probe synthesis kit (Roche Diagnostics, Basel, Switzerland) and the primers Kleb_MP_F1 (5′-GAG CAA AGT TAC TGT TTC TAT GGA-3′) and Kleb_MP-R1 (5′-TGA GCC ATC TTT CAT CAA CC-3′) on the *K. pneumoniae* strain B 5055. Dot blot hybridization was performed according to the manufacturer's protocol on Hybond N+ nylon membranes (Amersham, Pharmacia Biotech, Piscataway, NJ, USA), and the hybridized probe was visualized by using the DIG nucleic acid detection kit (Roche Diagnostics).

Capsular K serotyping was performed at the World Health Organization International *Escherichia* and *Klebsiella* Reference Centre (Copenhagen, Denmark) by using standard methods. In brief, K-typing was conducted by counter current immunoelectrophoresis (CCIE) with a modified version of the method described by Palfreyman (*17*). An extract was used as antigen instead of a whole cell suspension; the extract use was a modification because it was only heated once for 1 h at 100°C before centrifugation (*18*). All isolates with negative or doubtful reactions in CCIE were investigated by the classic Quellung technique, and K-type nontypeable isolates were investigated for the presence (K+) or absence (K–) of a visible capsule by wet

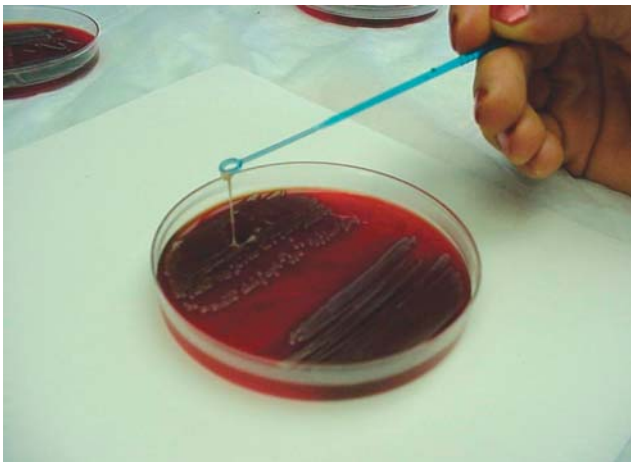

Figure 1. Mucoid phenotype of *Klebsiella pneumoniae.* When colonies were touched with a loop and the loop lifted vertically from the surface of the agar plate, mucoid isolates adhered to the loop as it was lifted from the plate. (Figure first presented at the 36th annual conference of the Infectious Diseases Society of America, Denver, Colorado, USA, 1998.)

mount microscopy with India ink. Lipopolysaccharide O typing was performed by a previously described inhibition ELISA (*19*).

Aerobactin production was demonstrated by a cross-feeding bioassay that used *Escherichia coli* strain LG 1522 (*20*). The clinical isolates were grown overnight in M9 broth containing the iron chelator 2-2′ dipyridyl. Strains were spotted onto hardened dipyridyl minimal agar plates. After 18 hours' incubation at 37°C, satellite growth of the indicator strain LG 1522 around the spots indicated aerobactin production.

Lethality in Mice

A standard inoculum of 1–2 × 10^7 bacteria in the logarithmic phase of growth from blood culture isolates from each study site was injected intravenously into the tail vein of C57/BL6J black, female mice, 8–12 weeks old. Two mice (Jackson Laboratories, Bar Harbor, ME, USA) were inoculated with each strain. Mortality of the mice was observed at 24 hours postinjection. The animal experiments were approved by the Institutional Review Board of the Veterans Affairs Medical Center, Pittsburgh, Pennsylvania, USA.

Pulsed-Field Gel Electrophoresis (PFGE)

The genotypic relationships of *K. pneumoniae* bloodstream isolates were determined by using PFGE. PFGE was performed by means of the CHEF-DR II system (Bio-Rad, Richmond, CA, USA) with use of the restriction endonuclease Xba I (New England Biolabs, Beverly, MA, USA). DNA was subjected to electrophoresis for 22 hours at 14°C in a 1% agarose gel at 6 V/cm with a linear gradient pulse time of 5–35 seconds. The gels were analyzed by using the Gel Doc 2000 software (Bio-Rad).

Statistics

Patient demographics and laboratory data were entered into PROPHET Statistics version 6.0 (AbTech Corporation, Charlottesville, VA, USA). The χ^2 or Fisher test was used to compare categorical variables. Continuous variables were compared by using the *t* test or the Mann-Whitney test. Multivariate analysis was used to determine which risk factors for mouse lethality by univariate analysis were independently significant.

Results

Serotypes of *K. pneumoniae* Bacteremic Strains

During the study period, 455 episodes of *K. pneumoniae* bloodstream infection occurred; 141 community-acquired bloodstream isolates were available and were tested for K serotype. Three isolates were not encapsulated, and 20 were nontypeable. Forty-seven different capsular serotypes were found in the remaining strains; K1 (16%; 23/141), K2 (11%; 16/141), and K54 (9%; 12/141) were the most common serotypes.

One hundred percent (23/23) of K1 serotype strains, 94% (15/16) of K2 serotype strains, and 100% (12/12) of K54 serotype strains were from Taiwan or South Africa. Forty-seven percent (23/49) of isolates from patients with community-acquired pneumonia and 50% (7/14) of isolates from patients with invasive syndromes possessed the K1 or K2 serotype. In comparison, 12% (9/78) isolates from patients with other manifestations of community-acquired *K. pneumoniae* bacteremia had the K1 or K2 serotype ($p<0.001$).

Seventy-three randomly chosen hospital-acquired bloodstream isolates from the same multinational study were tested for K serotype. One isolate was not encapsulated, and 5 isolates were nontypeable. In contrast to the predominance of K1 serotypes in community-acquired infections, 1% (1/75) of hospital-acquired isolates were K1 ($p = 0.0015$). The percentage of K2 serotypes in hospital acquired isolates (9%, 7/75) was similar to that observed in community-acquired isolates (11%, 16/141) ($p = 0.76$). The hospital-acquired K1 isolate was from Taiwan; 4 hospital-acquired K2 isolates were from Africa, 2 were from the United States, and 1 was from Taiwan. In contrast to the predominance of K1 and K2 serotypes in community-acquired pneumonia (47%, 23/49 isolates), these serotypes were significantly less likely to occur in nosocomial pneumonia (11%, 2/18 cases; $p = 0.022$).

Relationship between Capsular Serotype and Lethality in Mice

When community-acquired strains only were examined, highest lethality was observed with strains of K1 serotype (mouse deaths 65%) and K2 serotype (mouse deaths 81%). In contrast, lethality for community-acquired strains of other serotypes was significantly lower (23% died; $p<0.001$). The proportion of deaths in mice injected with community-acquired strains of serotype K54 was 9%.

When hospital-acquired strains were examined, mouse mortality was only 8%. The only lethal strains were K1 and K17 serotypes. None of the 7 hospital-acquired strains of serotype K2 proved lethal to mice.

When both community- and hospital-acquired strains were assessed together, lethality was significantly higher for strains of K1 (mouse deaths 67%) and K2 serotype (mouse deaths 59%) than for strains of other serotype (mouse deaths 20%) ($p<0.001$). The lethality of all strains of serotype 54 was 12.5%.

Lipopolysaccharide O Typing

Of 195 isolates which underwent O typing, 30% (58/195) were group O1, 17% (34/195) were O2, 8%

(16/195) were O3, 2% (3/195) were O4, 3% (6/195) were O5, 1% (2/195) were O2ac, 7% (13/195) were O–, and 32% (63/195) were O+ but not typable. No strains of O group O3, O4, O5, O2ac or O– were lethal to mice. In contrast, 21% (12/58) of O1 strains, 26% (9/34) of O2 strains, and 37% (23/63) of nontypeable O+ strains were lethal to mice.

Relationship between Mucoid Phenotype and Type of Infection

The mucoid phenotype was observed in 93% (13/14) of strains from patients with invasive disease, in 67% (33/49) of strains from patients with community-acquired pneumonia, and in 28% (22/78) of strains from patients with other sites of community-acquired infection ($p<0.001$). Mucoid strains of community-acquired bacteremic *K. pneumoniae* were exclusively found in Taiwan and South Africa; 56% (45/80) and 60% (21/35) of Taiwanese and African strains, respectively, were mucoid, whereas no mucoid strains were detected in community-acquired bacteremic strains from other countries (Table 1). In Taiwan and South Africa, mucoid strains predominated in community-acquired strains (57%, 66/115) compared with hospital-acquired strains (18%, 7/38) (Table 2).

In Taiwan and South Africa, community-acquired pneumonia was due to mucoid strains of *K. pneumoniae* in younger patients with no serious underlying disease, while nonmucoid strains predominated in older patients and those with serious underlying disease (Table 3). In both countries combined, 94% (29/31) strains from community-acquired pneumonia patients with no serious underlying disease had the mucoid phenotype compared to 19% (3/16) strains from patients with serious underlying disease ($p<0.001$). All isolates (13/13) from patients in Taiwan or South Africa with the invasive syndrome of liver abscess, meningitis, or endophthalmitis had the mucoid phenotype (Table 4).

The proportion of deaths in mice injected with mucoid strains (69% of mice died) was strikingly higher than that occurring in mice injected with nonmucoid strains (3% mice died) ($p<0.001$). There was no association between human deaths and the presence of a mucoid strain (39% died) or a nonmucoid strain (35% died) ($p>0.20$). In a multivariate model, increased severity of illness score when first evaluated ($p = 0.0001$), but not infection with a mucoid strain, country of origin, or history of alcoholism ($p>0.20$ for all) was associated with human deaths.

Association between Phenotypic Evidence of Mucoidity and Presence of *rmpA* Gene

Phenotypic evidence of mucoidity as judged by the definition in the methods section ("a string-like growth observed to attach to the loop as it was lifted from the plate") (Figure 1) was highly correlated with the presence of the *rmpA* gene. Of 77 mucoid isolates, 86% (66/77) were *rmpA* gene positive, and 14% (11/77) were *rmpA* gene negative. Of 137 nonmucoid isolates, 93% (128/137) were negative for the *rmpA* gene and 7% (9/137) were *rmpA* positive.

Relationship between Aerobactin Production and Type of Infection

The presence of the *rmpA* gene and phenotypic evidence of aerobactin production were closely correlated. Ninety-six percent of *rmpA* gene–positive isolates were aerobactin producers; aerobactin was produced by 2% of isolates that were *rmpA* gene–negative.

Associations between aerobactin production and type of infection were similar to those between the mucoid phenotype and type of infection. Only 6% (4/62) strains from patients in countries other than Taiwan and South Africa were aerobactin producers. In Taiwan and South Africa, 66% of patients with community-acquired pneumonia and 85% of patients with the invasive syndrome had aerobactin-producing strains, in comparison with 42% of patients with other community-acquired infections and 16% of patients with hospital-acquired strains (Table 2).

Relationship between Mucoid Phenotype, Capsular Serotype, and Lethality in Mice

When both community-acquired and hospital-acquired strains were considered together, 77% (36/47) of isolates of

Table 1. Disease type by virulence factor*

Source of organism and type of infection	K1 or K2 serotype, %	Mucoid phenotype, %	Aerobactin producer, %	Mouse mortality rate, %†
Taiwan				
Community-acquired	28 (22/80)	56 (45/80)	54 (43/80)	46
Hospital-acquired	17 (2/12)	25 (3/12)	33 (4/12)	29
South Africa				
Community-acquired	46 (16/35)	60 (21/35)	63 (22/35)	52
Hospital-acquired	16 (4/25)	16 (4/25)	8 (2/25)	0
Rest of world				
Community-acquired	4 (1/26)	0 (0/26)	4 (1.26)	0
Hospital-acquired	5 (2/36)	8 (3/36)	8 (3/36)	0

*Note the virtual absence of putative virulence factors in strains from the rest of the world, other than Taiwan and South Africa. See Results for p values.
†2 mice were tested for each available strain.

Table 2. Strain source and virulence factors, Taiwan and South Africa*

Infection type	K1 or K2 serotype, %	Mucoid phenotype, %	Aerobactin producer, %	Mouse mortality rate, %†
Community-acquired pneumonia	49 (23/47)	68 (32/47)	66 (31/47)	47
Invasive syndrome	54 (7/13)	100 (13/13)	85 (11/13)	82
Other community-acquired	15 (8/55)	38 (21/55)	42 (23/55)	36
Hospital-acquired	16 (6/38)	18 (7/37)	16 (6/38)	7

*Note that strains from patients in Taiwan and South Africa with community-acquired pneumonia or the invasive syndrome (liver abscess, endophthalmitis, meningitis) were more likely to have the putative virulence factors than hospital-acquired strains. See Results for p values.
†2 mice were tested for each available strain.

serotypes K1 and K2 were found to be mucoid. Of the other 45 serotypes, 24% (40/167) were mucoid ($p<0.001$).

However, none of the mice inoculated with nonmucoid K1 or K2 serotype strains died, compared with 81% of mice inoculated with mucoid K1 or K2 serotype strains ($p<0.001$). Just 4% of mice inoculated with nonmucoid strains of serotypes other than K1 or K2 died, compared with 44% mice inoculated with mucoid organisms of serotypes other than K1 or K2 ($p<0.001$).

When the parameters of mucoid phenotype, serotypes K1 and K2, and country of origin were assessed in the multivariate model of lethality to mice, mucoid phenotype was strongly associated with the death of mice ($p = 0.001$). Presence of serotypes K1 and K2 approached statistical significance ($p = 0.05$).

PFGE

PFGE was performed on strains of the same serotype. Dendrograms of organisms of serotype K1 are shown in Figure 2 and dendrograms of serotype K2 in Figure 3.

Discussion

We have been able to evaluate geographic differences in community-acquired *K. pneumoniae* infections by studying consecutive patients with community-acquired *K. pneumoniae* bacteremia from 7 different countries during the same period. It could be hypothesized that patient characteristics are primarily responsible for these differences. For example, genetic predilections (susceptibility of Asians to liver abscess), underlying diseases (for example, higher prevalence of chronic hepatitis B virus infection in Taiwan), social factors (different foods or cultural practices), and economic factors (for example, access to healthcare, antimicrobial drug usage) may be responsible for the different manifestations of serious *K. pneumoniae* infection observed in different regions. Despite these other possibilities, our experimental studies suggest that the differences in clinical features arise from differences in the virulence of individual microorganisms.

In particular, we found that strains with K1 or K2 serotype, strains with a mucoid phenotype, and strains that are capable of aerobactin production are rarely found to cause substantial infection in patients from study hospitals outside Taiwan and South Africa. Strains with such virulence characteristics were more likely to cause community-acquired infections than hospital-acquired infections. When strains with these virulence characteristics were inoculated into mice, deaths exceeded 80% compared with mortality rates of <5% in mice inoculated with strains lacking these characteristics.

Additionally, by PFGE, we found genetically related strains possessing all 3 virulence characteristics (Figures 2, 3). Taiwanese investigators have debated whether *K. pneumoniae* strains that cause liver abscess in Taiwan are clonally related (*1*,*4*,*6*,*7*,*21*). Genetic relatedness in *K. pneumoniae* strains that cause community-acquired pneumonia has not been previously described. However, we have found that genotypically related organisms were responsible for bacteremic community-acquired pneumonia due to *K. pneumoniae* and sometimes both pneumonia and liver abscess or meningitis. Whether *K. pneumoniae* is spread from person to person, whether related strains are acquired from common sources, or whether virulent strains arise from a common ancestor remains to be determined.

Much prominence has been placed in the past on the role of capsule in the pathogenesis of *K. pneumoniae* infec-

Table 3. Underlying disease and virulence factors in community-acquired *K. pneumoniae* pneumonia*

Country/condition	K1 or K2 serotype, %	Mucoid phenotype, %	Aerobactin production, %	Mouse mortality rate, %†
South Africa	50 (12/24)	75 (18/24)	67 (16/24)	58
No underlying disease	63 (12/19)	89 (17/19)	79 (15/19)	78
Underlying disease‡	0 (0/5)	20 (1/5)	20 (1/5)	0
Taiwan	48 (11/43)	65 (15/23)	57 (13/23)	35
No underlying disease	75 (9/12)	100 (12/12)	83 (10/12)	50
Underlying disease‡	18 (2/11)	18 (2/11)	27 (3/11)	18

*Note that patients with no underlying disease were more likely to be infected by strains with the putative risk factors than were elderly patients or patients with serious underlying disease. See Results for p values.
†2 mice were tested for each available strain.
‡Underlying disease was defined as presence of end-stage liver or renal failure, metastatic malignancy, neutropenia, or age >70.

Table 4. Mucoid strains in patients with liver abscess, endophthalmitis, or meningitis associated with community-acquired *Klebsiella pneumoniae* bacteremia*

Country	K1 or K2 serotype, %	Mucoid phenotype, %	Aerobactin producer, %	Mouse mortality rate, %†
Taiwan	50 (6/12)	100 (12/12)	85 (10/12)	81
South Africa	100 (1/1)	100 (1/1)	100 (1/1)	100

*Mucoid strains are highly lethal to mice.
†2 mice were tested for each available strain.

tions. Capsular types K1 and K2 have been regarded as particularly virulent (*22*). Taiwanese researchers have found that serotype K1 is frequently associated with community-acquired *K. pneumoniae* bacteremia (*3*,*23*). We have also confirmed that serotypes K1 and K2 occur more frequently in isolates from community-acquired infections in Taiwan and South Africa than from hospital-acquired isolates in these countries or elsewhere (Table 1).

The degree of virulence conferred by a particular K antigen may be related to the mannose content of the capsular polysaccharide. Capsular types with high virulence in animal models (for example, K2) lack mannose-α-2/3-mannose structures found in capsular types of lower virulence (*24*). The mannose-α-2/3-mannose structures are recognized by a surface lectin of macrophages, which mediate complement and antibody-independent phagocytosis. Strains that lack these sequences (for example, those with the K2 antigen) may not be recognized by macrophages, and hence phagocytosis may not take place. Furthermore, surfactant protein A (the main protein component of lung surfactant) enhances the phagocytosis by alveolar macrophages of strains that bear mannose-α-2/3-mannose structures in their capsule, but not strains which lack the mannose structure (*25*).

Some strains belonging to the K2 serotype are not as virulent as others (*26*). Thus, factors other than capsule may also be important for virulence. Although previous authors did not find any markers for these differences (*26*), we found that mucoid strains of K1 or K2 serotype were more virulent to mice than nonmucoid strains of the same serotype. No mouse inoculated with nonmucoid K1 or K2 serotype strains died, compared to 81% mice inoculated with mucoid K1 or K2 serotype strains ($p<0.001$). Multivariate analysis of variables related to mouse mortality rates showed that mucoidity was more closely associated with death than was capsular serotype. Contrary to popular belief, the biochemical nature of the mucoid phenotype may be unrelated to capsular polysaccharide but rather related to extracapsular polysaccharide (*27*). A previous study has shown that the mucoid phenotype may be due to a gene designated *rmpA* (regulator of mucoid phenotype) (*28*). In another mouse model, a mutant carrying this gene was 1,000-fold more virulent than an isolate without the gene. Extracellular polysaccharides may protect mucoid strains of *K. pneumoniae* from phagocytosis by neutrophils and from serum killing by complements (*27*).

We found that the mucoid phenotype frequently coexists with aerobactin production. The growth of bacteria in host tissues is limited not only by host defense mechanisms but also by its supply of available iron. The supply of free iron in the host milieu may be extremely low; many bacteria attempt to secure their supply of iron in the host by secreting high-affinity iron chelators called siderophores. Aerobactin is a hydroxamate-type siderophore occasionally found in *Klebsiella* strains. *K. pneumoniae* strains that produce aerobactin were more virulent in our mouse model, whereas strains not producing this siderophore were less likely to be; additionally, patients with severe community-acquired infection were more likely to be infected by aerobactin-producing strains. In another mouse model, transfer

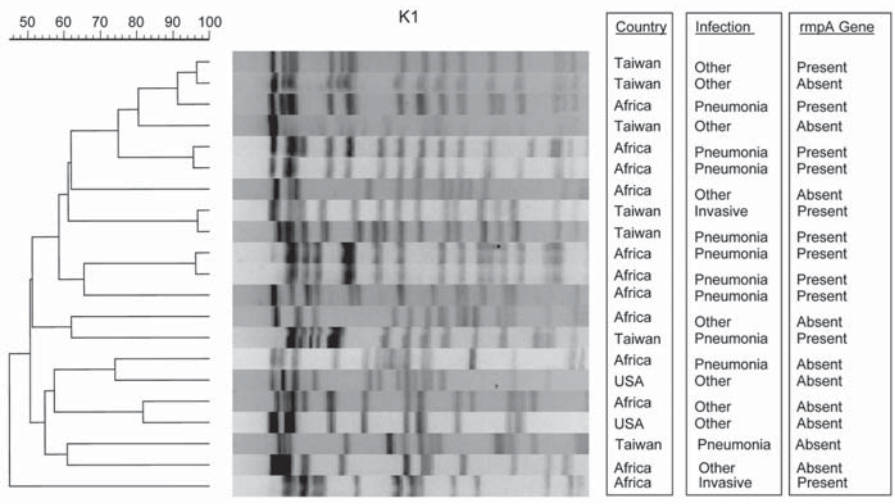

Figure 2. Pulsed-field gel electrophoresis of bacteremic *Klebsiella pneumoniae* isolates of serotype K1.

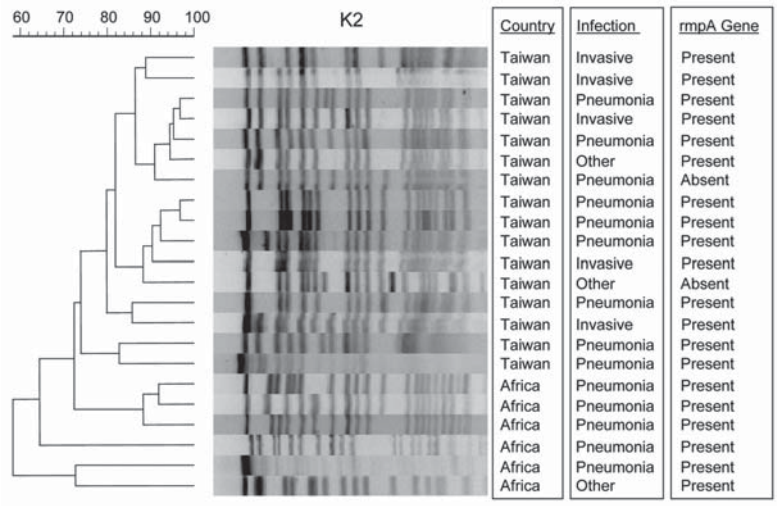

Figure 3. Pulsed-field gel electrophoresis of bacteremic *Klesiella pneumoniae* isolates of serotype K2.

of a recombinant plasmid harboring the genes for aerobactin and its receptor enhanced the virulence of an otherwise avirulent strain by 100-fold (*29*). The strong association found in this study between mucoid phenotype (*rmpA* positive isolates) and aerobactin production suggests that the 2 virulence characteristics might be genetically coupled on a large virulence plasmid, as has previously been demonstrated (*28*). We have not yet determined which of these 2 virulence factors is more important.

Strains harboring these virulence factors appear to be more frequent in certain geographic regions, and this may explain geographic differences in manifestation of community-acquired *Klebsiella* infections. The evolutionary genetics of *K. pneumoniae* have never been explored. To our knowledge, we have been the first to find clones bearing multiple virulence characteristics that are responsible for life-threatening community-acquired *K. pneumoniae* pneumonia in otherwise healthy persons. Investigation into the mechanisms of virulence in *K. pneumoniae* could lead to preventive measures (such as vaccination) in high-risk parts of the world.

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Dr Yu is professor of medicine at the University of Pittsburgh. His research interests include Legionnaires' disease, pneumonia, and antibiotic-resistant bacteria.

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Address for correspondence: Victor L. Yu, Special Pathogens Laboratory, 1401 Forbes Ave, Pittsburgh, PA 15240, USA; email: vly@pitt.edu

Antimicrobial Drugs and Community-acquired Methicillin-Resistant *Staphylococcus aureus*, United Kingdom

Verena Schneider-Lindner,*† J. A. Delaney,*† Sandra Dial,†‡ Andre Dascal,‡ and Samy Suissa*†

We report results of a case–control study of the association between receipt of antimicrobial agents and diagnosis of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) in the United Kingdom. Eligible adults, selected from the General Practice Research Database, had no previous diagnosis of MRSA, no hospitalization in the past 2 years, and ≥2 years of follow-up recorded in the database. For 2000–2004, we identified 1,981 MRSA case-patients and 19,779 matched control-patients. The odds ratios (ORs) and 95% confidence intervals (CIs) of MRSA diagnosis for patients who were prescribed 1, 2–3, or ≥4 antimicrobial drugs were 1.57 (CI 1.36–1.80), 2.46 (CI 2.15–2.83), and 6.24 (CI 5.43–7.17), respectively. Risk for community-acquired MRSA increased with number of antimicrobial drug prescriptions, appeared to vary according to antimicrobial drug classes prescribed the previous year, and was highest for quinolones (OR 3.37, CI 2.80–4.09) and macrolides (OR 2.50, CI 2.14–2.91).

Methicillin-resistant *Staphylococcus aureus* (MRSA) was detected in the United Kingdom in 1961, only months after methicillin introduction (*1–3*). Since then, MRSA has become a common cause of nosocomial infections worldwide (*1–3*). In 1993, MRSA infections emerging in the community were reported (*1*). MRSA infections acquired in the community differ from those acquired in the hospital with respect to their epidemiology and the characteristics of the causative MRSA strains (*1,2,4–6*).

The prevalence of colonization with MRSA has been established in various community populations (*7–9*). However, patient characteristics and risk factors for clinically significant MRSA infections acquired in the community have so far been described for specific outbreaks (*10,11*) or case series without an adequate population-based control group (*12,13*).

Nosocomial MRSA is associated with antimicrobial drugs and specific antimicrobial drug classes (*14–16*). The role of antimicrobial drugs in community-acquired MRSA is less clear. A recent study of 34 case-patients with community-acquired MRSA in Alaska showed that case-patients were more likely than control-patients to have received antimicrobial agents in the year before the outbreak (*10*). Whether risk for community-acquired MRSA differs according to exposure to agents from different antimicrobial drug classes is not clear. We therefore sought to describe the association between exposure to antimicrobial drugs and a subsequent diagnosis of MRSA, including exposure to individual antimicrobial drug classes.

Methods

We conducted our retrospective case–control study by using the General Practice Research Database (GPRD). This primary care database contains the diagnostic, laboratory test, and prescribing records of ≈3.2 million patients from >400 general practices in the United Kingdom. The GPRD is used extensively for research on drugs (*17,18*) and has also been used for research on infectious diseases (*19,20*).

Case-Patients and Control-Patients

Eligible participants were ≥18 years of age, had no previous diagnosis of MRSA, no hospitalization in the past 2 years, and ≥2 years of follow-up recorded in the GPRD. We excluded persons who had been recently hospitalized to ensure that we studied patients with community-acquired rather than community-onset MRSA.

*McGill University Health Center, Montreal, Quebec, Canada; †Royal Victoria Hospital, Montreal, Quebec, Canada; and ‡Sir Mortimer B. Davis–Jewish General Hospital, Montreal, Quebec, Canada

We identified as case-patients all persons with a first clinical diagnosis of MRSA from January 1, 2000, through December 31, 2004. To include all possible codes that a general practitioner could use to diagnose MRSA in the GPRD, we considered the following Read Clinical Classification codes (now National Health Service Clinical Terms) to represent a diagnosis of MRSA: 4JP..00 (methicillin-resistant *Staphylococcus aureus* positive), SP25800 (MRSA infection of postoperative wound), and ZV02A00 ([V]MRSA-multiple resistant *Staphylococcus aureus* infection carrier). Because the most frequently entered code (4JP..00) does not explicitly differentiate between infection and colonization, we were unable to determine from the codes whether most patients in this study were infected or colonized. The date of the MRSA diagnosis was used as the index date for each case. Microbiologic test results were not used to identify case-patients with MRSA because results for such testing were not systematically available in the GPRD.

For each case-patient, we randomly selected 10 control-patients also from the GPRD, matched by general practice and age (±2 years). To control for calendar time, we assigned all control-patients their corresponding case-patient's index date. Control-patients had to fulfill the same exclusion criteria as case-patients.

Exposure

For patients in each group, we determined exposure to antimicrobial drugs 30–365 days before the index date. To avoid the possibility of protopathic bias, we excluded antimicrobial drug prescriptions made during the 29 days prior to the index date. We classified the number of antimicrobial drugs prescribed for each patient during this period into 4 categories: 0 (unexposed), 1, 2–3, and ≥4 prescriptions. For the same period, we defined 7 mutually exclusive categories for classes of antimicrobial drugs according to their British National Formulary (BNF) code (*21*): penicillins (5.1.1), cephalosporins (5.1.2), tetracyclines (5.1.3), macrolides (5.1.5), sulfonamides (5.1.8), quinolones (5.1.12), and an additional category of all other antimicrobial drugs. The "other antimicrobial drugs" category represented BNF antimicrobial drug categories that are infrequently prescribed (such as clindamycin) or for which the antimicrobial drug is part of a combination prescription listed in a nonantimicrobial drug BNF category.

Covariates

We adjusted for age, sex, smoking, and obesity (body mass index >30) as relevant demographic and life style factors possibly associated with exposure to antimicrobial drugs and MRSA infections. We further controlled for a series of known risk factors and concurrent conditions diagnosed during the 2 years before the index date: heart disease (myocardial infarction, congestive heart failure), stroke, peripheral vascular disease, chronic obstructive pulmonary disease, liver disease, skin diseases (intertrigo, eczema, psoriasis), renal failure, cancer, autoimmune diseases (lupus, rheumatoid arthritis), previous infection with *Clostridium difficile*, and previous infection with *S. aureus* (susceptible to methicillin). These conditions were defined according to diagnostic codes. We defined diabetes according to prescribed insulin or a clinical diagnosis in the 2 years before the index date. Finally, because of its immunosuppressive effect, we considered oral prednisone (defined according to prescriptions) prescribed during the 1 year before the index date to be a potentially confounding drug.

Data Analysis

For each year of the study period, we calculated the incidence rate of first MRSA diagnosis. The numerator consisted of the number of eligible case-patients each year; the denominator was all members of the GPRD population who were registered with general practices that met GPRD quality control standards and who were ≥18 years of age during that year.

We used conditional logistic regression to estimate the odds ratio (OR) of the association between antimicrobial drug prescriptions and a subsequent diagnosis of MRSA (*22*). For a rare outcome like MRSA, the OR is an approximation of the rate ratio. To obtain adjusted ORs, we repeated the analyses with covariates included in the regression model. In a separate analysis for the number of antimicrobial prescriptions, we included variables that represented the 4 categories we defined for the number of prescriptions. For our analysis of association according to different class of antimicrobial drug, we used a separate statistical model that contained variables for all 7 categories that represented the different classes of antimicrobial drugs.

In a sensitivity analysis of the 3 Read Clinical Classification codes of our case definition, we determined the association between antimicrobial drugs and MRSA diagnosis in 3 different models. In each model, we included only the subset of case-patients who had the appropriate code and their corresponding control-patients. We also included the covariates in each of the 3 models to obtain adjusted ORs.

We used SAS version 9.1.3 (SAS Institute, Cary, NC, USA) for all analyses. We obtained approval from the Scientific and Ethical Advisory Group of the GPRD and the McGill University Health Center Research Ethics Board at the Chest Hospital.

Results

A total of 3,408 patients had a first diagnosis of MRSA in the GPRD during the study period. After exclusion of patients who did not fit the other criteria, 1,981 (58.1%) remained eligible for our study, for which we identified

19,779 matching control-patients. The MRSA diagnosis was recorded as medical code 4JP..00 for 85.5%, code SP25800 for 8.8%, and code ZV02A00 for 5.7% of case-patients.

During the study period, the annual number of case-patients with MRSA in the GPRD-based population rose from 332 to 484 (Figure). Average incidence of MRSA infections during the study period was 15.2 cases per 100,000 persons per year. The median age of case-patients during the study period was 74 years (interquartile range 59–83 years).

Overall, concurrent conditions were diagnosed more frequently for case-patients than for control-patients, and oral prednisone was prescribed more often for case-patients (Table 1). Smoking status did not differ significantly (adjusted OR 1.06, 95% CI 0.95–1.17), but more case-patients than control-patients were recorded as obese (OR 1.27, 95% CI 1.10–1.45).

Exposure to any antimicrobial drug in the 30–365 days before index date, regardless of drug class and number of prescriptions, was associated with ≈3-fold risk of a MRSA diagnosis when compared with lack of such exposure (OR 2.61, 95% CI 2.36–2.89, Table 2). Among case-patients, 38.9% had not received any antimicrobial drug prescription during this period.

The association of antimicrobial drugs and MRSA was stronger for persons who had received more prescriptions of any class of antimicrobial drug (Table 2). A substantial proportion of case-patients (25.7%) had received ≥4 prescriptions. This larger number of prescriptions was associated with a ≈6-fold increase in the risk for MRSA compared with the risk for persons who had not received any antimicrobial drug prescriptions (OR 6.24, 95% CI 5.43–7.17).

Individual classes of antimicrobial drugs were differentially associated with a diagnosis of MRSA (Table 3). The association was strongest for macrolides and quinolones; adjusted ORs were 2.50 (95% CI 2.14–2.91) and

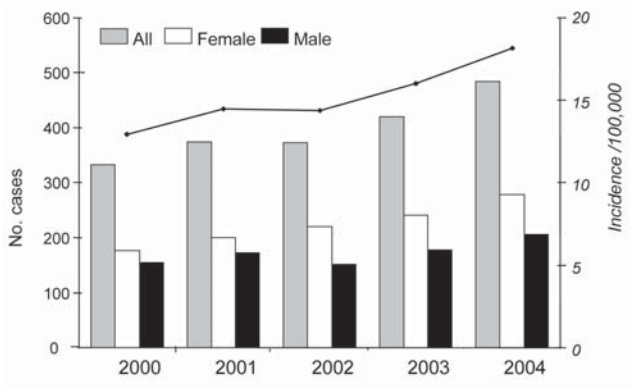

Figure. Annual number of all study participants with methicillin-resistant *Staphylococcus aureus* (MRSA) recorded for the first time in the General Practice Research Database (GPRD) and no hospitalization in the past 24 months (vertical bars). The annual incidence rate of MRSA per 100,000 adults in the GPRD is indicated by the line above the bars. Data from the GPRD, United Kingdom, 2000–2004

Table 1. Characteristics of patients with (case-patients) and without (control-patients) a diagnosis of community-acquired methicillin-resistant *Staphylococcus aureus**

Characteristic	Case-patients (N = 1,981), no. (%)	Control-patients (N = 19,779), no. (%)	Crude OR	Adjusted OR†	95% CI
Age, y (SD)	69.4 (18.4)	69.2 (18.5)	1.07	1.08	1.07–1.12‡
Male	864 (43.6)	8,429 (42.6)	1.05	1.08	0.98–1.20
Concurrent conditions					
Diabetes	247 (12.5)	1,195 (6.0)	2.27	1.70	1.44–2.00‡
MI and heart failure	117 (5.9)	563 (2.8)	2.22	1.42	1.13–1.80‡
Stroke	95 (4.8)	212 (1.1)	4.64	4.09	3.13–5.35‡
Peripheral vascular disease	49 2.5)	92 (0.5)	5.49	3.65	2.49–5.35‡
COPD	117 (5.9)	465 (2.4)	2.68	1.45	1.13–1.85‡
Skin diseases (intertrigo, eczema, psoriasis)	196 (9.9)	1,332 (6.7)	1.53	1.19	1.01–1.41‡
Renal failure	65 (3.3)	188 (1.0)	3.64	2.53	1.88–3.50‡
Cancer	42 (2.1)	183 (0.9)	2.39	2.01	1.39–2.91‡
Liver disease	6 (0.3)	8 (0.04)	7.50	6.69	2.17–20.66‡
Autoimmune diseases	17 (0.9)	93 (0.5)	1.84	1.27	0.72–2.25
Clostridium difficile	9 (0.5)	15 (0.1)	6.20	5.33	2.13–13.35‡
S. aureus susceptible to methicillin	8 (0.4)	14 (0.1)	5.71	2.98	1.13–7.83‡
Oral prednisone use	216 (10.9)	1,163 (5.9)	1.99	1.11	0.93–1.33
Lifestyle factors					
Smoking	718 (36.2)	6,365 32.2)	1.21	1.06	0.95–1.17
Obesity	323 (16.3)	2,253 (11.4)	1.54	1.27	1.10–1.47‡

*Data from the General Practice Research Database, United Kingdom, 2000–2004. OR, odds ratio; CI, confidence interval; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease.
†Adjusted for all other variables in the table and exposure to antimicrobial drugs in the 30–365 days period before the index date.
‡p<0.05.

Table 2. Risk for infection with community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA)*

No. prescriptions of antimicrobial drugs	Case-patients (N = 1,981), no. (%)	Control-patients (N = 19,779), no. (%)	Crude OR	Adjusted OR†	95% CI
0 (reference)	770 (38.9)	12,821 (64.8)	1	1	
≥1	1,211 (61.1)	6,958 (35.2)	2.98	2.61	2.36–2.89
1	328 (16.6)	3,306 (16.7)	1.69	1.57	1.36–1.80
2 or 3	373 (18.8)	2,389 (12.1)	2.73	2.46	2.15–2.83
≥4	510 (25.7)	1,263 (6.4)	7.27	6.24	5.43–7.17

*For persons prescribed ≤1 antimicrobial drug in the 30–365 days before their index date, relative to risk for MRSA infection in persons with no prescriptions during the same period. Data from General Practice Research Database, United Kingdom, 2000–2004. OR, odds ratio; CI, confidence interval.
†Adjusted for all variables in Table 1.

3.37 (95% CI 2.80–4.09), respectively. This association means that risk for an MRSA diagnosis triples for persons who received ≥1 prescription of a quinolone, regardless of how many other antimicrobial drugs were prescribed for this patient during the 30–365 days before the diagnosis. We could not establish an association between tetracycline prescriptions and MRSA in our study.

The results of our sensitivity analysis for diagnostic codes are presented in Table 4. Despite some variation in the estimated OR between the different methods of coding the MRSA diagnosis, we found an association with antimicrobial drugs, regardless of the code used to record the MRSA diagnosis.

Discussion

This study provides evidence of an association between previous antimicrobial drug prescriptions and a diagnosis of MRSA in the community. This association appears to be dose-dependent and to vary according to antimicrobial class; it is particularly strong for previous exposure to fluoroquinolones and macrolides. A substantial proportion of case-patients, however, were not prescribed antimicrobial drugs in the year before MRSA diagnosis. Persons who had concurrent conditions and persons who were obese were at higher risk for MRSA.

The clear dose-response relationship and the differential associations across antimicrobial drug classes support an association of antimicrobial drugs and MRSA in the community. This association is consistent with nosocomial MRSA, in which the use of specific antimicrobial drugs is linked to antimicrobial resistance. Previous studies have found fluoroquinolones, macrolides, and cephalosporins to be associated with nosocomial MRSA (*14–16,23*). Moreover, a dose-dependent association exists between exposure to antimicrobial drugs and nosocomial MRSA on the patient level and on the hospital level (*16*).

In a US surveillance study, incidence of community-acquired MRSA was 25.7 case-patients per 100,000 in Atlanta and 18.0 case-patients per 100,000 in Baltimore, findings that are highly consistent with ours (*12*). In that study, a considerable proportion of cases occurred in persons >65 years of age. In our study, compared with previous outbreak reports (*10,11*), case-patients were older and had more concurrent conditions. This finding is likely because we did not include any cases in children and, in contrast to reports of outbreaks, our cases are sporadic and thus less prone to be reported.

Similarly, specific occupation-related risk factors (e.g., abrasions, crowded housing) are likely to be more prevalent in a study based on military beneficiaries compared with a study based on the general population. This could explain the higher incidence of MRSA infections found in such a study (*13*) than in ours.

Our study population was a representative sample of the UK general population (*17*). The use of the GPRD therefore enables the examination of a large number of MRSA infections diagnosed by general practitioners in the community. However, the GPRD has some limitations for the study of

Table 3. Risk for infection with community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA)*

Class of antimicrobial drug	Case-patients (N = 1,981), no. (%)	Control-patients (N = 19,779), no. (%)	Crude OR	Adjusted OR†	95% CI
No prescription (reference group)	770 (38.9)	12,821 (64.8)	1	1	
Cephalosporins	27 13.6 ()	994 (5.0)	2.01	1.85	1.57–2.19
Macrolides	32 (16.2)	1,04 (5.2)	2.70	2.50	2.14–2.91
Penicillins	540 (27.3)	3,104 (15.7)	1.67	1.56	1.39–1.76
Other antimicrobial drugs	204 (10.3)	1,615 (8.1)	2.02	1.90	1.61–2.24
Sulfonamides	260 (13.1)	1,114 (5.8)	1.77	1.74	1.48–2.04
Tetracyclines	77 (3.9)	461 (2.3)	1.14	1.09	0.83–1.43
Quinolones	218 (11.0)	434 (2.2)	3.81	3.37	2.80–4.09

*For persons prescribed different classes of antimicrobial drugs in the 30–365 days before their index date, relative to risk for MRSA infection in persons with no prescriptions during the same period. Data from General Practice Research Database, United Kingdom, 2000–2004. OR, odds ratio; CI, confidence interval.
†Adjusted for prescriptions from other antimicrobial drug classes in the 30–365 days period before the index date and all variables in Table 1.

Table 4. Sensitivity analysis for the clinical code used to define methicillin-resistant *Staphylococcus aureus* (MRSA)*

	Case-patients		Control-patients				
Clinical code†	N (%)	% Exposed	N	% Exposed	Crude OR	Adjusted OR‡	95% CI
Any code	1,981 (100)	61.1	19,779	35.2	2.98	2.61	2.36–2.89
4JP..00	1,735 (85.5)	62.2	17,327	35.7	3.05	2.66	2.39–2.97
SP25800	157 (8.8)	60.5	1,570	32.7	3.34	2.99	2.06–4.33
ZV02A00	113 (5.7)	47.8	1,122	30.5	2.09	1.98	1.30–3.01

*Risk for MRSA diagnosed in the community for persons with any number of antimicrobial drug prescriptions (exposed) in the 30–365 days before their index date relative to risk for MRSA for persons with no prescriptions during the same period. Data from 4 different analyses of a matched case-control study of patients listed in the General Practice Research Database, UK, 2000–2004. OR, odds ratio; CI, confidence interval.
†4JP..00, MRSA positive; SP25800, MRSA infection of postoperative wound; ZV02A00, [V]MRSA multiple-resistant *Staphylococcus aureus* infection carrier.
‡Adjusted for all covariates in Table 1.

an infectious disease. We lacked information on severity and site of MRSA infection and on patient lifestyle characteristics (e.g., incarceration, intravenous drug use). We also lacked information on molecular characteristics of the MRSA strains and thus cannot exclude the possibility that MRSA was diagnosed in cases that did not result from between-patient spread within the community, but rather from secondary exposure to the hospital environment through family members, visitation, or employment. However, the importance of the lack of microbiologic information on the causative MRSA strains may be questioned because it no longer enables a distinction to be made between community and nosocomial MRSA strains (*24*).

The clinical codes that we used for our case definition are likely to be specific, but they may lack sensitivity; some MRSA infections that would have met the Centers for Disease Control and Prevention's case definition of infection may not be captured with the clinical codes. This lack of sensitivity may affect risk factors for MRSA that we observed in this study as well as the strength of the association between antimicrobial drugs and later MRSA diagnosis.

To our knowledge, MRSA infections in the GPRD have not been previously studied, although infectious diseases in this database have been, such as acute respiratory infections (*19*), urinary tract infections (*20*), *C. difficile* infections (*25*), pneumonia (*26*), and sexually transmitted infections (*27*). Disease codes for clinically relevant outcomes in the GPRD have been validated (*17*); however, MRSA has not been included in such studies.

In previous UK community studies, ≈0.8% of elderly participants were colonized with MRSA (*9*,*28*). In a meta-analysis, the pooled MRSA colonization rate was 1.3% (*8*). In persons without prior health care contacts, the rate was 0.2% (*8*). We report an incidence rate of MRSA diagnosis in the community that is too low to be consistent with these prevalence figures, probably because general practitioners are unlikely to screen asymptomatic patients for colonization with MRSA.

The results of our sensitivity analysis of the 3 diagnostic codes we used to define these MRSA cases suggest that antimicrobial drugs promote both MRSA infection and colonization. The association between antimicrobial drugs and MRSA diagnosis was weaker in case-patients with a diagnosis of carrier status and stronger in those with a postoperative wound infection. The association observed in the patients who had their diagnosis recorded with the most frequently used code (4JP..00) appeared to be more similar to those coded as postoperative wound infections (SP25800) than to those coded as carriers (ZV02A00). This finding supports the hypothesis that active infections are more likely than colonization to be recorded in a general practice database. Therefore, separating risk factors for acquiring MRSA from risk factors for increased severity of infection with MRSA using the approach of this study may be difficult.

We did not consider antimicrobial drugs that were prescribed in the 30 days before MRSA diagnosis. With this exposure definition, we prevent mistaking prescriptions issued for treatment of the infection as causes of the infection (protopathic bias [*29*]). This distinction is relevant for MRSA, for which the diagnosis is likely delayed due to outstanding microbiologic test results or likely made after failure of empirical treatment with antimicrobial drugs. Therefore, we may have wrongly classified as unexposed some persons whose exposure to antimicrobial drugs in fact preceded the MRSA infection. Any bias resulting from this exposure definition will be toward the null hypothesis and thus will weaken the effect of antimicrobial drugs as promoters of MRSA infections.

To minimize the chances of overlooking any important confounders, we adjusted our analyses for age, sex, lifestyle factors, and a broad range of concurrent conditions. That antimicrobial drug prescriptions are a marker for an important unknown confounder is remotely possible. However, to substantially bias our results, such a confounder would need to be strongly related to both the prescription of antimicrobial drugs and the diagnosis of MRSA but unrelated to our study covariates.

Further support for our results comes from the ecologic association between fluoroquinolone use and nosocomial MRSA found in an intervention study (*30*) and a quasi-experimental study (*31*). Similar to nosocomial MRSA (*16*,*23*), the use of specific antimicrobial drugs in the community may cause selection pressures that favor

the acquisition of resistance in *S. aureus* on the community level.

In conclusion, the role of antimicrobial drugs in MRSA diagnosed in the community appears to be similar to their role in nosocomial MRSA. Therefore, appropriate use of antimicrobial drugs, in addition to traditional infection control measures, may be a strategy to not only control nosocomial MRSA (*32*), but also to limit the incidence of community-diagnosed MRSA infections.

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Dr Schneider-Lindner is a PhD student in the Department of Epidemiology, Biostatistics and Occupational Health at McGill University. Her research focus is pharmacoepidemiology.

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Address for correspondence: Samy Suissa, Division of Clinical Epidemiology, Royal Victoria Hospital, 687 Pine Ave West, Ross 4.29, Montreal, Quebec, Canada H3A 1A1; email: samy.suissa@clinepi.mcgill.ca

Antiretroviral Therapy during Tuberculosis Treatment and Marked Reduction in Death Rate of HIV-Infected Patients, Thailand[1]

Somsak Akksilp,* Opart Karnkawinpong,* Wanpen Wattanaamornkiat,* Daranee Viriyakitja,† Patama Monkongdee,‡ Walya Sitti,* Dhanida Rienthong,† Taweesap Siraprapasiri,†‡ Charles D. Wells,§ Jordan W. Tappero,§‡, and Jay K. Varma§‡

Antiretroviral therapy (ART) is lifesaving in patients with advanced HIV infection, but the magnitude of benefit in HIV-infected patients receiving tuberculosis (TB) treatment remains uncertain, and population-based data from developing countries are limited. We prospectively collected data about HIV-infected TB patients from February 2003 through January 2004 in Ubon-ratchathani, Thailand. During 12 months, HIV was diagnosed in 329 (14%) of 2,342 patients registered for TB treatment. Of patients with known outcomes, death during TB treatment occurred in 5 (7%) of 71 who received ART and 94 (43%) of 219 who did not. Using multivariate analysis, we found a large reduction in the odds of death for patients receiving ART before or during TB treatment (odds ratio, 0.2; 95% confidence interval, 0.1–0.5), adjusting for CD4 count, smear status, co-trimoxazole use, and treatment facility. ART is associated with a substantial reduction in deaths during TB treatment for HIV-infected TB patients in Thailand.

Tuberculosis (TB) is one of the most common opportunistic infections and causes of death in HIV-infected persons (*1*). In developing countries, many HIV-infected persons frequently receive the diagnosis of HIV infection or AIDS after first having TB diagnosed at a health facility (*1*). The proportion of HIV-infected TB patients who die during TB treatment is high: an estimated 6%–39% die during TB treatment in sub-Saharan Africa (*2*,*3*). Deaths occurring in the first few months after TB diagnosis are more likely TB related, whereas deaths occurring later are more likely to be attributable to other HIV-related illnesses (*4*–*7*).

Thailand has experienced a severe TB/HIV syndemic, i.e., 2 diseases acting synergistically to cause excess illness and death (*8*). Almost 600,000 persons are currently HIV infected, and >90,000 TB cases are estimated to occur annually (*2*,*9*). One fourth of persons in whom AIDS was diagnosed first have TB, and an estimated 12% of TB cases in Thailand are HIV associated, although the proportion is as high as 40% in some provinces (*10*).

Antitretroviral therapy (ART), which uses highly active combinations of drugs, improves survival in HIV-infected persons (*11*,*12*). HIV-infected persons in Thailand now have widespread access to ART, but physicians often do not prescribe it to HIV-infected TB patients because of concerns about drug-drug interactions, overlapping toxicities, immune reconstitution syndrome, and pill burden. Expert groups and the World Health Organization (WHO) also recommend that public health programs make treatment of TB the first priority and ideally begin ART after TB treatment is tolerated and $CD4^+$ T-lymphocyte (CD4) count is measured (*13*). Several studies have now documented that ART reduces the likelihood of death during TB treatment of HIV-infected TB patients, but these studies relied on retrospective data collection, occurred outside

*Ministry of Public Health, Ubon-ratchathani, Thailand; †Ministry of Public Health, Nonthaburi, Thailand; and ‡Thailand Ministry of Public Health—Centers for Disease Control and Prevention Collaboration, Nonthaburi, Thailand; and §Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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routine public health programs, or involved resource-rich countries without large TB or HIV epidemics (*14–18*). In this study, we analyzed data from a prospective, population-based surveillance system to estimate the benefit of ART on reducing mortality during TB treatment in HIV-infected TB patients living in rural Thailand.

Methods

Setting

Ubon-ratchathani is a large, predominantly rural province in northeastern Thailand with a population of 1.7 million persons. The rate of reported TB cases is 145/100,000 persons, and HIV prevalence in women attending public antenatal clinics was 0.6% (in 2004). Treatment of TB or HIV is offered by 25 health facilities, including 20 Ministry of Public Health (MOPH) hospitals, 3 private hospitals, 1 military hospital, and 1 MOPH outpatient TB and HIV referral clinic. Except for those who are seriously ill, TB and HIV patients are managed in outpatient specialty clinics at these facilities.

Data Collection

In 2003, the US Centers for Disease Control and Prevention (CDC) began collaborating with the MOPH and Ubon-ratchathani Province on a special project to enhance surveillance, monitoring, evaluation, and treatment of TB, HIV-associated TB, and multidrug-resistant TB (MDR TB) in a project known as the Thailand TB Active Surveillance Network. For all patients with a diagnosis of TB in any of the 25 participating healthcare facilities, public health staff recorded standardized epidemiologic data, collected sputum specimens for laboratory testing (including staining for acid-fast bacilli [AFB], mycobacterial culture, species identification, and drug-susceptibility testing), and offered HIV counseling and testing.

Sputum specimens were collected for AFB staining and culture at the beginning of TB treatment. Specimens were cultured at an MOPH laboratory in the province on Ogawa (February–March 2003) or Lowenstein-Jensen agar (April 2003–January 2004) by using standard methods, and isolates were sent to the MOPH national reference laboratory for drug-susceptibility testing. Public health staff from the TB program collected patient data prospectively from routine medical and laboratory records, recorded data in a modified version of the standard national TB register, and entered data into an electronic database.

Patient Population

All persons registered for TB treatment, regardless of their final diagnosis, were considered TB patients, consistent with WHO guidelines (*19*). We restricted our analysis to TB patients who had laboratory confirmation of HIV infection and who were registered for TB treatment from February 2003 through January 2004. Patient outcomes were only recorded through the end of TB treatment, which was usually 6 months after treatment initiation; no data about outcomes were recorded after the end of TB treatment.

For TB treatment, patients received standardized regimens, consistent with WHO guidelines; new (not previously treated) patients received isoniazid, rifampin, ethambutol, and pyrazinamide (*18*). HIV-infected TB patients were referred to HIV-related care and treatment, but individual physicians used their own clinical judgment about measuring CD4 count, providing opportunistic infection prophylaxis or ART, and managing other clinical conditions. When measured, CD4 counts were usually checked within the first month of TB treatment. Thai MOPH guidelines recommend that HIV-infected patients with CD4 <200 cells/mm^3 receive co-trimoxazole and stavudine, lamivudine, and nevirapine (known as "GPO-vir"); in patients with TB, efavirenz is recommended instead of nevirapine.

Definitions

Standard WHO definitions were used to categorize patients according to previous TB treatment history, type of TB (sputum smear–positive, pulmonary; sputum smear–negative, pulmonary; extrapulmonary), and treatment outcome. Consistent with WHO guidelines, we classified all deaths occurring during TB treatment, whether the cause was known or not, as a TB death (*18*).

We classified ART use according to whether ART was begun before TB treatment, begun during TB treatment, or not taken during TB treatment. We classified co-trimoxazole use as either taken or not taken during TB treatment. No data on interruptions of ART or co-trimoxazole treatment were collected; for the purposes of surveillance, any patient already taking or started on ART or co-trimoxazole was considered to be taking it throughout TB treatment. We stratified CD4 count (cells/mm^3) as <50, 50–99, 100–199, and ≥200.

Data Analysis

For descriptive analysis, all patients were included. For univariate and multivariate analysis of risk factors for death, we restricted our analysis to TB patients with an outcome of cured, completed, failed, or died; we excluded patients who defaulted on treatment or transferred out, because their final treatment outcome was not known. Patients with an outcome of failure were combined with those who were cured or completed treatment, since all 3 groups were known to have survived the first 6 months of TB treatment. We calculated relative risk (RR) for factors associated with death in patients with all forms of TB and in the subset of patients with pulmonary, sputum smear–positive TB.

For multivariate analysis, we calculated adjusted odds ratios (OR) for factors associated with death by using logistic regression. Variables were chosen based on ≥1 of the following: statistical significance ($p<0.05$) in univariate analysis, biologic plausibility, or previously published evidence. Because 41% of patients had data missing for CD4 count, we performed several analyses to explore the impact of missing CD4 count on our final model estimates, including the following: 1) classifying patients with unknown CD4 as a separate strata in analysis; 2) recoding patients with unknown CD4 as having CD4 <50 cells/mm^3; 3) recoding patients with unknown CD4 as CD4 >200 cells/mm^3; and 4) excluding all patients with unknown CD4 from the analysis. Because rates of default were high and cases of default may actually be deaths, we also recoded cases of default as death and repeated all multivariate analyses. In analyses for which there were no outcomes in some CD4 strata, we log-transformed the CD4 count and modeled it as a continuous variable (*20*). The protocol for this project was reviewed by the Thailand MOPH and CDC and the study was found to be surveillance and public health program implementation and not human subjects research requiring oversight by an institutional review board.

Results

From February 2003 through January 2004, 2,342 patients were registered for TB treatment in Ubon-ratchathani Province. Of these, 225 (10%) were known to be HIV infected before their TB diagnosis. Of the remaining 2,117 patients, 1,626 (77%) received HIV pretest counseling, 680/1,626 (42%) agreed to HIV testing, and 104/680 (15%) were found to be HIV infected. In all, 329 (14%) of the 2,342 total TB patients were either known to be HIV infected before TB diagnosis (225; 68% of all TB/HIV patients) or were identified as HIV infected after testing through the TB program (104; 32% of all TB/HIV patients).

The median age of the 329 HIV-infected TB patients was 32 years (range 10 months–68 years), 112 (34%) were female, and 307 (93%) had new TB cases (Table 1). TB was classified as sputum smear–positive in 120 (36%), sputum smear–negative in 107 (33%), and extrapulmonary in 102 (31%). CD4 count was unavailable or not performed in 134 (41%). Of the 195 patients with CD4 results available, the median CD4 count was 53 cells/mm^3 (range 1–873); 93% had CD4 <200 cells/mm^3.

Sputum cultures were performed in 145 (64%) of 227 patients with pulmonary TB, including 93 (78%) of 120 with sputum smear–positive TB and 52 (49%) of 107 with sputum smear–negative TB (Table 2). Of the 93 patients whose sputum smears were positive and who had a culture performed, 65 (70%) grew *Mycobacterium tuberculosis* (MTB); of these, 4 (6%) isolates were resistant to at least isoniazid and rifampin, i.e., MDR TB. Of the 52 sputum smear–negative patients with a culture performed, only 3 (6%) were culture positive, and none exhibited MDR TB.

Table 1. Characteristics of HIV-infected patients with tuberculosis (TB), Ubon-ratchathani, February 2003 through January 2004

Characteristic	Patients (N = 329), no. (%)
Female	112 (34)
Median age, y (range)	32 (0.8–68)
Type, location of TB	
Sputum smear-positive, pulmonary	120 (36)
Sputum smear-negative, pulmonary	107 (33)
Extrapulmonary	102 (31)
Category of TB	
New	307 (93)
Treatment after interruption, failure, default, or relapse	11 (4)
Other	11 (3)
HIV status known before TB diagnosis	225 (68)
CD4 count (cells/mm^3)	
<50	95 (29)
50–99	39 (12)
100–199	36 (11)
≥200	25 (8)
Unknown	134 (41)
Received co-trimoxazole during TB treatment	225 (68)
Received antiretroviral therapy (ART)	
Before TB diagnosis	30 (9)
During TB treatment	45 (14)
Not prescribed ART	254 (77)
Among patients receiving ART, regimen prescribed (n = 75)	
Stavudine/Lamivudine/Nevirapine	38 (51)
Stavudine/Lamivudine/Efavirenz	35 (47)
Other	2 (2)
Treatment outcome	
Cured	61 (19)
Completed	126 (38)
Failure	4 (1)
Default	31 (9)
Transfer	4 (1)
Change of diagnosis	4 (1)
Died	99 (30)

Before TB treatment, 30 (9%) patients were receiving ART; an additional 45 (14%) patients began ART during TB treatment; and the remaining 254 (77%) patients did not receive ART before or during TB treatment. In 40 of the 45 patients who began ART during TB treatment and in whom a date of starting ART was available, the median time between TB diagnosis and ART initiation was 93 days (range 0–170 days). Among all patients receiving ART, 38 (51%) received a combination regimen of stavudine, lamivudine, and nevirapine; 35 (47%) received efavirenz instead of nevirapine; and 2 (2%) were on other regimens. During TB treatment, 225 (68%) received co-trimoxazole.

Of all 329 patients, 187 (57%) were cured or completed TB treatment; 99 (30%) died during TB treatment. In

Table 2. Results of culture and susceptibility testing performed on HIV-infected patients with pulmonary TB, stratified by sputum smear–positive versus –negative results, Ubon-ratchathani, February 2003 through January 2004*

Characteristic	Smear-positive (n = 120), no. (%)	Smear-negative (n = 107), no. (%)
Sputum culture performed	93/120 (78)	52/107 (49)
Culture positive for nontuberculous mycobacteria	2/93 (2)	0/52 (0)
Contaminated	5/93 (5)	6/52 (12)
Culture negative	21/93 (23)	43/52 (83)
Culture positive for *Mycobacterium tuberculosis*	65/93 (70)	3/52 (6)
Multidrug-resistant TB	4/65 (6)	0/3 (0)
Previously treated	1/4 (25)	NA

*TB, tuberculosis; NA, not available.

the remaining 43 patients, treatment failed (for 4 patients) or the patient defaulted (a WHO term defined as missing at least 2 continuous months of treatment) (31 patients), transferred out (4 patients), or received a final diagnosis other than TB (4 patients). Of the 4 patients with MDR TB, 3 died and 1 was recorded as having failed treatment with final outcome not recorded.

In univariate analysis restricted to the 290 patients with an outcome of cured, completed treatment, failed treatment, or died, we analyzed several factors associated with death during TB treatment. For all TB patients, having an unknown CD4 count was associated with increased likelihood of death, and receiving co-trimoxazole or ART was associated with reduced mortality (Table 3). For ART, 5 (7%) of 71 patients who received ART died compared with 94 (43%) of 219 patients who did not receive ART (RR 0.2; 95% confidence interval [CI] 0.1–0.4; absolute risk reduction 36; number-needed-to-treat 2.8). For sputum smear–positive TB patients, results were similar; additionally, male patients were at higher risk for death than female patients (RR 2.3, 95% CI 1.1–4.7).

In multivariate analysis adjusted for CD4 count, smear status, hospital providing treatment, and co-trimoxazole use, ART remained strongly associated with reduced mortality during TB treatment (Table 4). The adjusted OR (aOR) for death in patients who received ART before or during TB treatment was 0.2 (95% CI 0.1–0.5) compared with that in patients who did not receive ART. Receiving co-trimoxazole was no longer significantly associated with reduced mortality. We found virtually identical results when we did the following: 1) restricted our analysis to only those patients who received ART during TB treatment compared with patients who did not receive ART during TB treatment; 2) restricted our analysis to previously untreated, non-MDR patients without nontuberculous mycobacteria; 3) coded patients with unknown CD4 as having CD4 >200 cells/mm^3, as having CD4 <50 cells/mm^3, or as missing (i.e., removed from the analysis). All analyses also produced essentially identical results when we reclassified cases of default as death.

When we restricted our analysis to sputum smear–positive patients, we found a similarly strong beneficial effect for ART. Because no deaths occurred in the group of smear-positive patients with CD4 >200 cells/mm^3, we modeled CD4 as a continuous variable. The aOR was 0.1 (95% CI 0.0–0.9). Results were similar when we recoded patients with unknown CD4 count as having CD4 equal to 50 cells/mm^3 (indicative of profound immunosuppression and imminent risk of death) or 250 cells/mm^3 (not eligible for antiretroviral treatment in many country guidelines because they are relatively immune competent). Because of small sample size, we were only able to perform univariate, not multivariate, analysis for the 57 culture-positive patients who had an outcome of cured, completed, failed, or died. One (8%) of 13 culture-positive patients receiving ART died compared with 17 (36%) of 47 culture-positive patients not receiving ART (RR 0.2, 95% CI 0.0–1.5; absolute risk reduction 28; number-needed-to-treat 3.6).

Because patients who died soon after TB diagnosis were also unlikely to have begun ART, we modeled the effect of ART after excluding 32 patients who died in the first month of beginning TB treatment (aOR 0.1, 95% CI 0.0–0.8) and the additional 16 patients who died in the second month (aOR 0.1, 95% CI 0.0–1.2). Results were similar when we recoded patients with unknown CD4 count as having CD4 equal to 50 cells/mm^3 or 250 cells/mm^3, except that the effect of ART was now statistically significant for the analysis excluding deaths within the first and second months (aOR 0.1, 95% CI 0.0–0.7 for unknown CD4 recoded as 50 cells/mm^3; aOR 0.1; 95% CI 0.0–0.9 for unknown CD4 recoded as 250 cells/mm^3).

We explored the effect of co-trimoxazole on mortality for the 218 patients who did not receive ART: 52 (38%) of 137 patients receiving co-trimoxazole died compared with 42 (51%) of 82 patients not receiving co-trimoxazole (RR 0.7, CI 0.6–1.0; absolute risk reduction 13; number-needed-to-treat 7.7). The association between co-trimoxazole and survival was not statistically significant when we excluded patients who died in the first month (RR 0.9, CI 0.5–1.5) or in the first 2 months (RR 1.2, CI 0.5–3.0) and when we limited the analysis to smear-positive patients (RR 0.8, CI 0.5–1.3). In multivariate analysis of patients who did not receive ART and adjusting for CD4 count, smear status, and hospital providing treatment, co-trimoxazole was not associated with survival (aOR 0.9, CI 0.5–1.9).

Table 3. Univariate analysis of risk factors for death among HIV-infected TB patients with outcomes of cured, completed, failed, or died, stratified by all patients versus pulmonary, smear-positive patients, Ubon-ratchathani, February 2003 through January 2004*

	All TB patients (N = 286)		Smear-positive TB patients (n = 104)	
Characteristic	Died, no./total (%)	RR (95% CI)	Died, no./total (%)	RR (95% CI)
Sex				
Male	69/193 (36)	1.2 (0.8–1.6)	33/72 (46)	2.3 (1.1–4.7)
Female	30/97 (31)	Ref	7/35 (20)	Ref
Age				
<18 y	4/19 (21)	0.6 (0.2–1.4)	0/1 (0)	0 (0–0)
18–34 y	60/168 (36)	Ref	26/60 (43)	Ref
≥35 y	35/103 (34)	1.0 (0.7–1.3)	14/46 (30)	0.7 (0.4–1.2)
Type, location of TB				
Sputum smear-positive, pulmonary	40/107 (37)	Ref	NA	NA
Sputum smear-negative, pulmonary	38/96 (40)	1.1 (0.8–1.5)	NA	NA
Extrapulmonary	21/87 (24)	0.7 (0.4–1.0)	NA	NA
CD4 count (cells/mm^3)				
≥200	1/22 (5)	Ref	0/8 (0)	Ref
100–199	5/33 (15)	3.3 (0.4–26.6)	2/12 (17)	Undefined
50–99	9/35 (26)	5.7 (0.8–41.6)	1/10 (10)	Undefined
<50	22/92 (24)	5.3 (0.8–37.0)	9/34 (27)	Undefined
Unknown	62/108 (57)	12.6 (1.9–86.3)	28/43 (65)	Undefined
Co-trimoxazole during TB treatment				
Received	57/208 (27)	0.5 (0.4–0.7)	21/71 (30)	0.6 (0.4–0.9)
Did not receive	42/82 (51)	Ref	19/36 (53)	Ref
Antiretroviral therapy before or during TB treatment				
Received	5/71 (7)	0.2 (0.1–0.4)	1/23 (4)	0.1 (0.0–0.7)
Did not receive	94/219 (43)	Ref	39/84 (46)	Ref
Sputum culture†				
Culture positive	18/60 (30)	Ref	18/59 (31)	Ref
Not culture positive	81/230 (35)	1.2 (0.8–1.8)‡	22/48 (46)	1.5 (0.9–2.5)‡
Culture negative	21/60 (35)	1.2 (0.7–2.0)‡	9/20 (45)	1.5 (0.8–2.7)‡

*TB, tuberculosis; RR, relative risk; CI, confidence interval; Ref, referent; NA, not applicable.
†"Culture positive" includes all patients with a sputum culture positive for *Mycobacterium tuberculosis* (MTB). "Not culture positive" includes any patients without a culture positive for MTB, regardless of whether they had a specimen sent for culture or not. "Culture negative" includes only patients with a sputum culture negative for MTB.
‡Compared with culture positive.

Discussion

In this prospective, population-based study from a rural province in northeastern Thailand, we documented a high rate of death in HIV-infected TB patients and a substantial reduction in the risk for death during TB treatment for patients receiving ART. In this population, TB occurred predominantly in persons with preexisting HIV diagnoses and low CD4 counts, and, as expected, the CD4 count was inversely related to death. These findings are more consistent with the epidemiology of TB in high-income countries, such as the United States, than that in sub-Saharan Africa, where studies have found that most TB and HIV have not previously been diagnosed in patients with HIV infection and that TB occurs across a broad spectrum of immune suppression (*21–26*). Although CD4 counts were not recorded for many patients in this study, we can infer from the high mortality in these patients that their CD4 counts were probably low. Research would be needed to document whether data from this evaluation are representative of other settings in Thailand or Southeast Asia. That TB occurred predominantly in persons with advanced, diagnosed HIV infection suggests that interventions targeted specifically at HIV-infected patients—such as early ART, treatment of latent TB infection, and earlier screening for and treatment of TB disease in household contacts of TB patients and during routine HIV care—are also needed to reduce incidence and mortality of HIV-associated TB.

Given the advanced immune suppression in this population, that ART improved survival during TB treatment is not surprising. The magnitude of benefit, however, was substantial. Treating 3 HIV-infected TB patients with ART in this population would translate into 1 life saved during TB treatment. In fact, co-trimoxazole, which is known to save lives during TB treatment in Africa (*27*), was not significantly associated with survival after adjusting for ART use. Co-trimoxazole protects AIDS patients against a wide range of infections that commonly occur in Thailand, including *Pneumocystis jirovecii* and *Toxoplasma* sp. (*28*). We were not able to demonstrate a survival benefit of co-trimoxazole in patients receiving ART or those not receiving ART. We do not know whether this is a true phenomenon or attributable to bias, misclassification, or small

Table 4. Multivariate analysis of risk factors for death among HIV-infected TB patients with outcomes of cured, completed, failed, or died and adjusted for site of treating facility,* Ubon-ratchathani, February 2003 through January 2004†

Characteristic	Adjusted OR (95% CI)
Antiretroviral therapy before or during TB treatment	0.2 (0.1–0.5)
Co-trimoxazole during TB treatment	1.1 (0.6–2.3)
CD4 count (cells/mm^3)	
≥200	Referent
100–199	5.5 (0.6–52.6)
50–99	9.3 (1.0–82.9)
<50	9.7 (1.2–78.4)
Unknown	29.9 (3.8–238.0)
Type, location of TB	
Sputum smear–positive, pulmonary	Referent
Sputum smear–negative, pulmonary	1.3 (0.7–2.6)
Extrapulmonary	0.5 (0.2–1.0)

*Data not shown.
†TB, tuberculosis; OR, odds ratio; CI, confidence interval.

sample size. Further studies are needed to evaluate the survival benefit of co-trimoxazole in HIV-infected TB patients in Thailand. Our sample size was too small to compare outcomes between patients receiving regimens with efavirenz versus nevirapine or to compare outcomes between patients who received ART during the first 2 months of TB treatment compared with those who received ART later.

Our evaluation reinforces the importance of providing TB patients with early access to HIV diagnosis and treatment, as recommended in WHO's Interim Policy on TB/HIV Collaborative Activities (*1*). In this project, we found several missed opportunities, including HIV testing of TB patients, measurement of CD4 count, and initiation of co-trimoxazole and ART. Educating providers about the life-saving benefits of ART in HIV-infected TB patients is a major priority, but more data from observational studies and clinical trials are needed to provide evidence-based guidance about the optimum timing of ART and the incidence and management of overlapping toxicities and immune-reconstitution syndrome.

This study has several major limitations. First, we do not know the reasons why patients did not receive ART. Many patients who did not receive ART were likely deemed too ill and, therefore, unlikely to benefit from ART. This bias would exaggerate the benefit of ART; survival would determine ART use, not the reverse. To address this issue, we controlled for 2 factors likely to predict when a patient would receive ART, such as CD4 count and hospital of care (a surrogate for physician preference or resources). Moreover, after excluding persons who died within the first 2 months of TB treatment, i.e., persons likely to receive minimal benefit from ART and likely to have been deemed too ill to receive ART, we still identified a substantial benefit for ART.

Another limitation is that not all TB patients underwent HIV testing, which could skew our population toward those patients most likely to have advanced immune suppression and, therefore, most likely to benefit from ART. Since these data were collected, rates of HIV testing of TB patients have increased substantially in Ubon-ratchathani, but the proportion identified as HIV infected remains similar to what it was in this study, suggesting that the number of HIV infections missed in this study population was small. This study was also based on surveillance and monitoring data from a public health program, which, though prospectively collected, necessarily relied on incomplete data, such as from routine medical records. Core data elements, such as CD4 count, were missing for many patients, and data about adverse events and causes of death, which are critical to assessing the risks of combined ART and TB treatment, were not collected. Low rates of culture positivity, particularly in smear-negative patients, leave open the possibility that some patients did not, in fact, have TB. Since this study, we have identified several reasons for the low yield of culture, including delayed transport times for specimens and inadequate specimen collection; efforts to improve these procedures have since been implemented. Even though many patients were not culture confirmed in this study, sputum smear–positive patients benefited strongly from ART, which suggests that misdiagnosis of TB is an unlikely explanation for our findings.

Major strides have been made in enhancing access to HIV treatment in the developing world. Nevertheless, as this study shows, deaths of patients with both TB and HIV remain high, and, even in a country such as Thailand with high rates of access to ART, few HIV-infected TB patients receive ART. Globally, measures to save lives of patients with both diseases have focused on making TB and HIV programs collaborate more closely. To that end, more data from these settings are urgently needed to convince policymakers in countries affected by this syndemic about the critical importance of rapidly expanding access to ART, particularly for HIV-infected TB patients.

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Dr Akksilp is a physician and public health official with the Thailand Ministry of Public Health. His interests include expanding access to diagnosis and treatment of TB, MDR-TB, and TB/HIV.

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Address for correspondence: Jay Varma, CDC/HIV, Box 68, American Embassy, APO, P 96546; email: jvarma@cdc.gov

Ongoing Genome Reduction in *Mycobacterium ulcerans*

Simona Rondini,*[1] Michael Käser,*[1] Timothy Stinear,†[2] Michel Tessier,‡ Cyrill Mangold,‡ Gregor Dernick,‡ Martin Naegeli,* Françoise Portaels,§ Ulrich Certa,‡ and Gerd Pluschke*

Elucidation of the transmission, epidemiology, and evolution of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer, is hampered by the striking lack of genetic diversity of this emerging pathogen. However, by using a prototype plasmid-based microarray that covered 10% of the genome, we found multiple genomic DNA deletions among 30 *M. ulcerans* clinical isolates of diverse geographic origins. Many of the changes appear to have been mediated by insertion sequence (IS) elements IS*2404* and IS*2606*, which have high copy numbers. Classification of the deleted genes according to their biological functions supports the hypothesis that *M. ulcerans* has recently evolved from the generalist environmental *M. marinum* to become a niche-adapted specialist. The substantial genomic diversity, along with a prototype microarray that covered a small portion of the genome, suggests that a genome-wide microarray will make available a genetic fingerprinting method with the high resolution required for microepidemiologic studies.

The study of genetic diversity within bacterial species has provided information on aspects such as virulence (*1*,*2*), antimicrobial drug resistance (*3*), epidemiology, and microbial evolution (*4*–*7*). For mycobacteria such as *Mycobacterium tuberculosis* and *M. ulcerans*, low intraspecies diversity limits the use of genetic fingerprinting techniques that are based on sequence diversity in selected genetic elements. For *M. tuberculosis*, *M. bovis*, and the various bacillus Calmette-Guérin daughter strains, genome-wide microarray analyses have identified large sequence polymorphisms (*4*,*8*–*10*). However, the complete genome sequence of an organism is required for the design of synthetic oligonucleotide or PCR product–based microarrays. When this information is not available, an alternative is a PCR product–based shotgun DNA microarray (*11*), which we developed further into a plasmid-based microarray. We used this method for the differential genomic analysis of *M. ulcerans*, a human pathogen for which the fully assembled and annotated genome sequence was not available at the time of the study.

M. ulcerans is the causative agent of Buruli ulcer, an infectious disease characterized by chronic necrotizing skin ulcers (*12*). Buruli ulcer is an emerging infectious disease found mostly in West African countries but also in tropical and subtropical regions of Asia, the Western Pacific, and Latin America (*13*). Genetic analyses suggest recent divergence of *M. ulcerans* from *M. marinum*, a well-known fish pathogen that can cause limited granulomatous skin infections in humans (*14*). One of the hallmarks of the emergence of *M. ulcerans* as a more severe pathogen is the acquisition of a 174-kb plasmid that bears a cluster of genes necessary for the synthesis of the polyketide toxin mycolactone. This toxin appears largely responsible for the massive tissue destruction seen in Buruli ulcer (*15*). The epidemiology and mode of transmission of *M. ulcerans* disease are not fully understood, partly because no molecular typing method with sufficiently high resolution for microepidemiologic analyses is available.

Standard molecular typing methods such as multilocus sequence typing, restriction fragment length polymorphism, and fingerprinting using variable number of tandem repeats have shown an apparent lack of genetic diversity of *M. ulcerans* within individual geographic regions, which is indicative of a clonal population structure. The genotyping technique that has shown the highest discriminatory power so far is based on the use of outward-directed primers spe-

*Swiss Tropical Institute, Basel, Switzerland; †Institut Pasteur, Paris, France; ‡F. Hoffmann-La Roche Ltd., Basel, Switzerland; and §Institute of Tropical Medicine, Antwerp, Belgium

[1]These authors contributed equally to this work.

[2]Current affiliation: Monash University, Melbourne, Victoria, Australia

cific for the insertion sequence (IS) IS*2404*, in combination with an oligonucleotide that targets a repeated GC-rich motif (*16*). Application of this method determined the resolution of 10 different *M. ulcerans* genotypes, which correspond to the geographic origin of the isolates. However, this level of resolution is not sufficient for microepidemiologic analyses. We hypothesized that, as for *M. tuberculosis* (*17*), deletional and insertional events mediated by repetitive sequence elements are a major mechanism for genomic variation in *M. ulcerans*. To test this hypothesis, we developed a plasmid-based microarray and analyzed genomic DNA from 30 *M. ulcerans* isolates of diverse origins.

Materials and Methods

Plasmid-Based DNA Microarray

From a shotgun clone library of strain Agy99, 352 *Escherichia coli* plasmids (pCDNA2.1, Invitrogen, Basel, Switzerland) were randomly selected. Each plasmid contained an *M. ulcerans* DNA fragment of ≈2.3–2.7 kb. Given a genome size of 5,806 kb (*18*), this set of plasmid inserts represents a theoretical genome coverage of ≈10%. Plasmid DNA was prepared by using a Biomek 2000 Workstation (Beckman Coulter, Krefeld, Germany) and dissolved at a concentration of 150 ng/μL in 3× SSC (20× SSC stock solution is 3 M sodium chloride, 0.2 M sodium citrate, pH 7.0). The DNA samples were loaded on a piezo-dispensing head that contained 24 channels and spotted onto glass slides coated with poly-L-lysine (Superfrost Plus, Menzel, Braunschweig, Germany) by using a Topspot spotter (Biofluidix, Freiburg, Germany). Slides were incubated at 4ºC overnight and rehydrated under 50%–60% humidity for 1 h at room temperature. The spots resulting from a volume of ≈1 nL had an average diameter of 270 μm and were 500 μm apart from each other. The microarray layout displayed 2 identical fields—for hybridization with 2 different probes—that consisted of 2 replicates each, both of which contained 32 controls and 352 plasmids.

Biotinylation of *M. ulcerans* Genomic DNA Fragments

M. ulcerans clinical isolates used in this study are listed in Figure 1. Bacterial pellets of about 60 mg (wet weight) were heat inactivated for 1 h at 95°C in 500 μL extraction buffer (50 mmol/L Tris-HCl, 25 mmol/L EDTA, 5% monosodium glutamate) and sequentially treated with lysozyme (2 h, 37°C, 17 M lysozyme) and proteinase K (overnight, 45°C, 0.3 M proteinase K in proteinase K buffer: 1 mmol/L Tris-HCl, 5 mmol/L EDTA, 0.05% sodium dodecyl sulfate [SDS], pH 7.8). After digestion the samples were subjected to bead beater treatment (Mikro-Dismembrator, Braun Biotech International, Berlin, Germany) with 300 μL of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) for 7 min at 3,000 rpm. DNA was extracted from the supernatants by phenol-chloroform (Fluka, Buchs, Switzerland) extraction and ethanol precipitation. Seven micrograms of *M. ulcerans* genomic DNA was digested with 3 U of *Sau*3A1 (New England Biolabs, Hitchin, UK) for 2 h at 37ºC and biotinylated according to Pollack et al. (*19*) using a BioPrime kit (Gibco/BRL, Gaithersburg, MD, USA). The biotinylated DNA was purified by using a Microcone YM-30 filter (Amicon/Millipore, Bedford, MA, USA), and its concentration was measured by optical density at 260 nm (GeneQuant spectrophotometer, Cambridge, UK).

Hybridization of Microarray Slides

Five micrograms of biotinylated DNA was mixed with 30 μg human Cot-1 DNA (Roche Applied Science, Indianapolis, IN, USA) and 100 μg yeast tRNA (Gibco/BRL). The hybridization mix was concentrated with a Speed Vac Concentrator System (Eppendorf, Basel, Switzerland), resolved in 3× SSC, 0.3% SDS, denatured for 3 min at 95ºC, and incubated for 30 min at 37ºC before hybridization. Microarray slides were cleaned with a nitrogen flow, exposed to UV light in a Stratalinker 2400 (Stratagene, La Jolla, CA, USA) at 650× 100 μJ, and heated for 5 min to 95ºC before application of 13 μL of the hybridization mix on each array field. Hybridization occurred for 20 h at 65ºC in a hydration chamber. Hybridized slides were washed once with 2× SSC, 0.03% SDS for 5 min at 65ºC, twice with 1× SSC for 5 min at room temperature, and finally with 0.2× SSC for 5 min at room temperature. The coloration step was performed with 2 mL staining solution containing 50% caseine, 1× maleic acid buffer (Roche Applied Science), and 2 μg Streptavidin Cy3 Fluorolink (Amersham, Piscataway, NJ, USA) for 30 min at room temperature, followed by additional washings for 5 min with 1× TBS (0.15 M

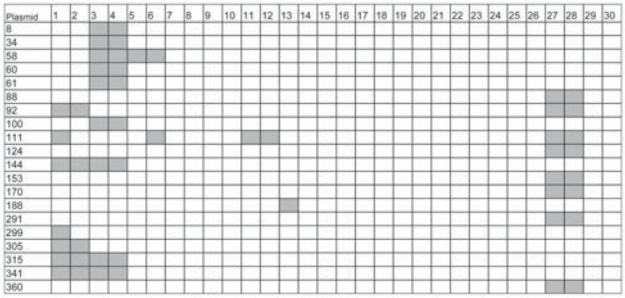

Figure 1. Distribution of outlier signals among the 30 *Mycobacterium ulcerans* isolates tested. *M. ulcerans* isolates: 1, 8756 Japan; 2, 980912 China; 3, 842 Suriname; 4, 7922 French Guiana; 5, 5147 Australia; 6, 5142 Australia; 7, 9549 Australia; 8, 9550 Australia; 9, 940339 Australia; 10, 8849 Australia; 11, 5151 Democratic Republic of Congo (DRC); 12, 5150 DRC; 13, 940511 Côte d'Ivoire; 14, 940662 Côte d'Ivoire; 15, 940815 Côte d'Ivoire; 16, 960658 Angola; 17, 960657 Angola; 18, 970680 Togo; 19, 970321 Ghana; 20, 970483 Ghana; 21, 970359 Ghana; 22, 940111 Benin; 23, 001441 Benin; 24, 940886 Benin; 25, 970104 Benin; 26, 940512 Benin; 27, 5143 Mexico; 28, 5114 Mexico; 29, 941331 Papua New Guinea (PNG); 30, 9537 PNG.

sodium chloride, 0.02 M Tris, pH 7.5) as well as 0.1× TBS and drying with a nitrogen flow. DNA of all 30 *M. ulcerans* strains was processed under identical conditions and hybridized at least twice, which yielded 4 sets of data for each strain. Human Cot-1 DNA and plasmid DNA without insert as well as a hybridization mix without DNA served as negative controls for hybridization. A 500-bp β-lactamase gene fragment and Cy3-labeled random oligonucleotides (Microsynth, Balgach, Switzerland) were used as positive controls and for estimation of the amount of spotted DNA.

Microarray Scanning and Data Evaluation

Images of the microarrays were acquired by using a laser microarray scanner (GenePix 4100A, Axon Instruments Inc., Foster City, CA, USA) with an excitation wave length of 532 nm, an emission wavelength of 570 nm, and standardized measurement parameters. The resulting image was analyzed by the software GenePix Pro 4.1 (Axon Instruments Inc.), which enabled assignments of mean intensity values used for data interpretation. To select spots to be included in the analysis of genomic diversity of *M. ulcerans* strains, replicates of 10 hybridizations were performed by using *M. ulcerans* Agy99 genomic DNA. All spots that showed a signal lower than twice that given by the negative control plasmid without insert were rejected, as were all spots for which coefficient of variation was >30%. Further analysis used 232 spots that had an average signal above the threshold and sufficient signal stability. For each plasmid, we calculated the average signal value, standard deviation, and coefficient of variation and assessed a signal ratio in comparison with the reference strain. Outlier spots with a ratio higher than U2 (U2 = upper quartile + 3× interquartile) were identified through a box-plot analysis.

Characterization of Large Sequence Polymorphisms

Microarray data that indicated the presence of a deletion were verified by PCR analysis, which used primer pairs that spanned the insertion sequences of the respective plasmids, the flanking regions, or both. The 5′ and 3′ limits of the confirmed genomic deletions with respect to the genome of strain Agy99 were determined by PCR analysis, which used multiple sets of primers complementary to flanking genomic regions. PCR analyses that bridged the genomic breakpoints were performed by using a long-range PCR polymerase mix (Fermentas, St Leon-Rot, Germany) according to the manufacturer's description. PCR products were cloned into pGEM-T (Catalys AG, Promega, Wallisellen, Switzerland) and sequenced using an ABI PRISM 310 genetic sequence analyzer (Perkin-Elmer, Waltham, MA, USA).

Results

Comparative Genomic Hybridization of *M. ulcerans* Isolates

We constructed a microarray based on a random selection of 232 *Escherichia coli* plasmids obtained from a shotgun sequence library of the *M. ulcerans* isolate Agy99 from Ghana. Genomic DNA hybridization signal intensities from 30 *M. ulcerans* clinical isolates of worldwide distribution (Figure 2) were compared with those obtained with strain Agy99. Box-plot analysis (Figure 3) identified plasmids that yielded outlier signals with respect to strain Agy99. For 19 of 20 plasmids, PCR analysis confirmed an association of the outlier signal with a genomic deletion. Only 1 low hybridization signal represented a false-positive result (p188 from strain 940511, Côte d'Ivoire; Figure 3). The number of confirmed outlier plasmids per isolate ranged from zero for most African isolates to 9 for isolates from Suriname and French Guiana (Figure 1).

Of the 19 plasmid inserts that yielded confirmed outlier signals, 3 (p111, p299, and p341) contained sequences from the virulence plasmid pMUM001 of *M. ulcerans*. Of the 16 plasmids derived from the *M. ulcerans* chromosome, some contained fragments that overlapped the same region (Figure 4). Hybridizing regions were almost identical for p60 and p61. Both plasmids yielded outlier values with the isolates from Suriname and French Guiana. A cluster of overlapping inserts was observed for p88, p153, and p360; these produced outlier values for both of the Mexican isolates. The same pattern was seen with p124 and p291, which have inserts that are located in close proximity to each other in the genome (Figure 3). These results from related inserts demonstrated the reproducibility of the differential hybridization analysis. Because the inserts p60–p61, p88–p153–p360, and p124–p291 were part of the same deletion in regions of difference (RDs) 4, 5, and 8, respectively; (Figure 4), altogether 12 chromosomal RDs were identified.

Figure 2. Distribution map of *Mycobacterium ulcerans* patient isolates used in this study (strain identity as listed in Figure 1).

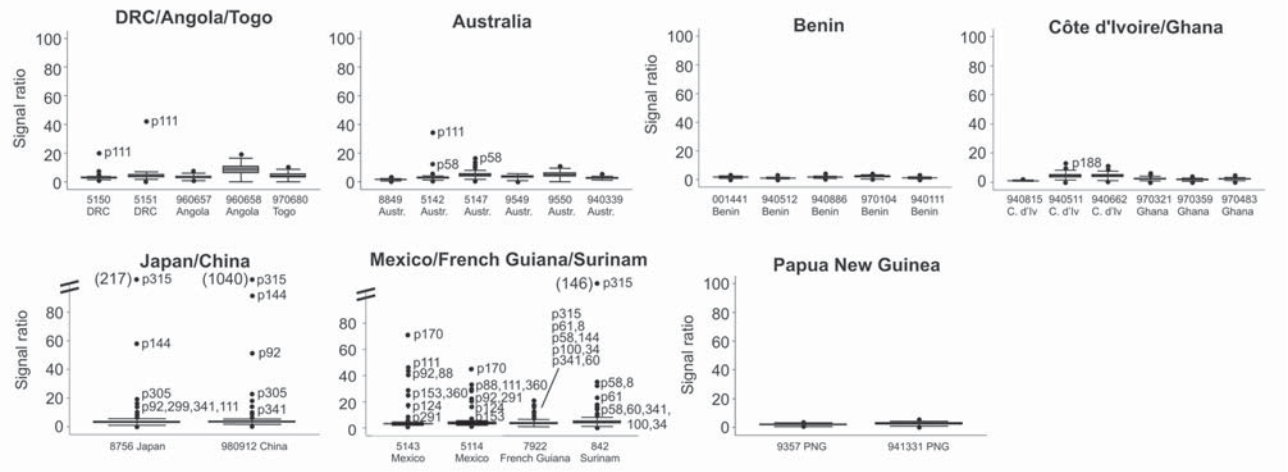

Figure 3. Box-plot analysis of the signal ratios obtained with genomic DNA of 30 *Mycobacterium ulcerans* strains identifying plasmids yielding outlier signals. Shown is the signal ratio in comparison with the African reference strain Agy99. The median of the ratios obtained is represented by the line in the center of the rectangular box. The 2 ends of the rectangles represent the upper quartile (UQ), which corresponds to the 75th percentile, and the lower quartile (LQ), which corresponds to the 25th percentile. The interquartile (IQ) is equal to UQ – LQ. The other 2 values shown are the maximum and minimum value of the data set: U1 = UQ + 1.5IQ; L1 = LQ – 1.5IQ. Double slashes represent discontinued range on the y-axis, values of the outliers beyond 100 in brackets. DRC, Democratic Republic of Congo; Austr., Australia; C. d'Iv, Côte d'Ivoire. PNG, Papua New Guinea.

Characterization of Genomic RDs

The 5′ and 3′ limits of the genomic deletions with respect to the genome of strain Agy99 were determined by PCR analysis that used multiple sets of primers complementary to plasmid inserts and to flanking genomic regions. The size of the deletions ranged from 1.8 kb to 53.1 kb (Table).

In 3 of the 12 RDs (RD3, 9, and 12), 2 distinct types of overlapping deletions (designated A and B) were observed, leading to a total of 15 large deletions. The overlapping deletions shared neither common 5′ nor 3′ end sequences. The strains from Australia had a 3.5-kb deletion in RD3; strains from Suriname and French Guiana had a slightly larger (3.8-kb) deletion. The isolates from Suriname and French Guiana had a larger (25.4-kb) deletion in RD9 than the isolates from Japan and China (17.7 kb). The largest deletion (53.1 kb) was designated RD12A and was observed in strains from Japan and China. Isolates from Suriname and French Guiana had a significantly smaller deletion in RD12 (35.2 kb). The 19.7-kb deletion 6 was found in isolates from 2 different regions (Mexico and Japan/China, respectively). All other deletions were observed in 2 isolates from the same region (Table).

To assess whether polymorphisms undetected by the microarray analysis would frequently occur in the identified RDs, we performed a detailed PCR analysis in all 30 *M. ulcerans* strains included in this study for 2 randomly selected RDs (RD5 and 12). We used 4 distinct primer pairs to span the insert sequence plus 5′ and 3′ flanking sequence stretches. For RD12, the PCR analysis confirmed the presence of a deletion in the 4 strains that had outlier signals in the microarray analysis, but no evidence for deletional polymorphism was obtained in the other strains. For RD5, PCR analysis confirmed the presence of a deletion in the 2 Mexican strains that had outlier signals (not shown). In addition, this PCR analysis identified the presence of an insertion in strains from Japan, China, Suriname, and French Guiana. The sequence of this 765-bp DNA insert was identical for all 4 strains. Its G+C content was 64%, and BLAST searches showed 98% identity with a sequence stretch of the *M. marinum* genome (www.sanger.ac.uk/cgi-bin/blast/submitblast/m_marinum) but no significant homology with sequences in the National Center for Biotechnology Information BLAST databases (www.ncbi.nlm.nih.gov/blast).

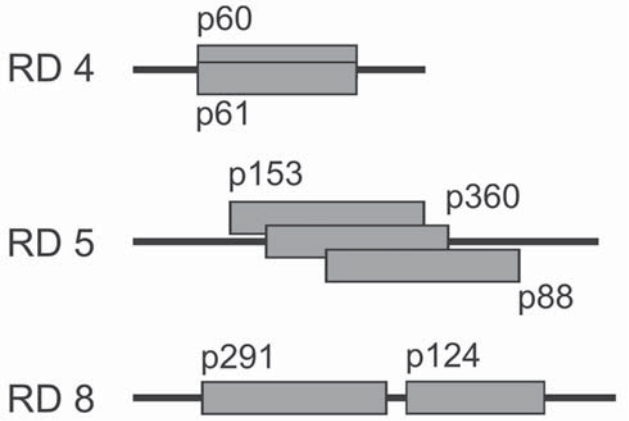

Figure 4. Positions of overlapping or adjacent plasmid inserts in regions of difference (RDs) 4, 5, and 8. Identical results retrieved by different plasmids with overlapping sequences or sequences in closest proximity demonstrate the reproducibility and reliability of the differential genomic hybridization method.

Table. Features of the 15 distinct deletions identified in *Mycobacterium ulcerans* isolates*

RD	Deletion no.	Plasmid no.	Origin	Size of deletion, kb	No. CDSs and pseudogenes
1	1	p8	SU/FG	11.1	14
2	2	p34	SU/FG	6.6–6.8	4
3	3A	p58	AU1/AU2	3.5	5
	3B	p58	SU/FG	3.8	6
4	4	p60, p61	SU/FG	1.8–2.4	2
5	5	p88, p153, p360	ME1/ME2	27.1–27.4	23
6	6	p92	JP/CH/ME1/ME2	19.7	24
7	7	p100	SU/FG	15.3	13
8	8	p124, p291	ME1/ME2	52.8–53.1	50
9	9A	p144	JP/CH	18.1	15
	9B	p144	SU/FG	25.4	20
10	10	p170	ME1/ME2	8.2–8.7	10
11	11	p305	JP/CH	4.6	7
12	12A	p315	JP/CH	53.1	50
	12B	p315	SU/FG	35.4–35.5	32

*RD, region of difference; CDSs, coding sequences; SU, Suriname; FG, French Guiana; AU, Australia; ME, Mexico; JP, Japan; CH, China.

Association of Deletions with Insertions

Of the 15 identified genome rearrangement events, 1 (deletion 3A observed in 2 Australian isolates) was found to be a deletion, with the genomic sequences flanking the 5′ and 3′ borders of the 3,451-bp deletion being directly joined (Figure 5). Analysis of the other 14 deletions showed that the loss of DNA in a given strain with respect to the genome of Agy99 was associated with the insertion of substituting sequences of varying sizes unrelated to the deleted regions. As an example, the larger (3,784-bp) deletion 3B found in the isolates from Suriname and French Guiana was associated with the insertion of an unrelated DNA fragment, which comprised the 1,368 bp of IS*2404* (*20*) plus an additional DNA stretch of 163 bp (Figure 5). For most of the other deletions, 1 of the 2 highly abundant insertion sequence elements (IS*2404* or IS*2606*) was situated in either the genomic sequences that flanked the deletion or that were in the deleted parts or in the substituting sequence stretches (as for deletion 3B).

Analysis of Coding Sequences and Pseudogenes in the Deleted DNA Sequences

The 15 deletions identified contained 52 pseudogenes and 185 predicted protein-coding sequences (CDSs), which represent 5.7% of the annotated 4,143 CDSs in the genome of the *M. ulcerans* strain Agy99 (*18*). The number of deleted CDSs and pseudogenes ranged from 2 (RD4) to 50 (RD8 and 12A) and averaged 18.6 per deletion (Table). CDSs were classified into 11 functional categories (*17*). When compared with the gene composition of the entire Agy99 genome, the following functional categories were overrepresented among the 185 deleted CDSs: insertion sequences, unique hypothetical genes, and predicted proteins involved in detoxification (Figure 6). Also overrepresented was the deletion of the 52 pseudogenes that contain frame shift mutations and premature stop codons or that are disrupted by an insertion sequence. In contrast, genes involved in intermediary metabolism, information pathways, and cell wall/cell processes were underrepresented among the deleted CDSs (Figure 6). Of the 185 deleted functional CDSs, 89 had orthologs with >50% amino acid sequence identity to proteins from the *M. tuberculosis* H37Rv genome. A tendency for gene categories to cluster within the RDs was found. RD2 comprises 2 PPE genes: RDs 1, 12A, and 12B are predominantly CDSs involved in lipid metabolism, and RDs 9A and 11 include mainly transcriptional regulators. However, overall *M. ulcerans* lineages from distinct geographic origin (Africa, Australia, Asia, South America, Mexico) did not differ markedly in the categories of deleted genes. RD8 (deleted in the Mexican strains) is particularly interesting because it contains a cluster of proteins of the mammalian cell entry *mce3* operon and associated regulators thereof. The transcriptional repressor, *Mce3R*, is considered to be an essential gene required for growth of *M. tuberculosis* (*21*). In addition, RD8 comprises a collection of CDSs of almost every functional category (online Appendix Table, available from www.cdc.gov/EID/content/13/7/1008-appT.htm). The spectrum of RD8-associated CDSs involved in detoxification included the multidrug transport protein *mmr*, the epoxide hydrolase *EphB*, the thiol peroxidase *Tpx*, and the alkyl hydroperoxide reductase C protein *AhpC*.

Although CDSs involved in intermediary metabolism were underrepresented among the deleted genes, 21 (42%) of deleted CDSs of this category were dehydrogenases (such as acyl-CoA short-chain alcohol, saccharopine, and aldehyde dehydrogenases), which are central enzymes in anaerobic metabolism (*22*) and important for survival in poorly oxygenated environments such as soil (*23*). In addition, other genes associated with anaerobic respiration, such as nitroreductases and electron transfer proteins, were found among the deleted CDSs.

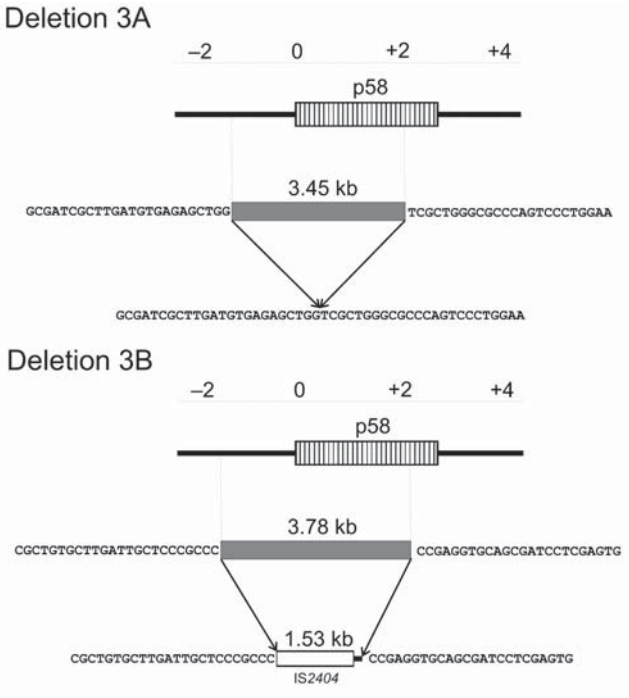

Figure 5. Two distinct deletions in region of difference 3. Although deletion 3A represents a mere deletion event, the larger deletion 3B is associated with an insertion event. Neither 5' nor 3' ends are identical in the 2 deletions.

Discussion

We describe the use of a plasmid-based DNA microarray for identifying large deletional and insertional genomic polymorphisms in a collection of 30 *M. ulcerans* strains of geographically diverse origin. A set of plasmids randomly selected from an *E. coli* shotgun library of *M. ulcerans* genomic DNA was spotted on microarray slides. This is a newly developed technology, highly suitable for situations in which the complete genome sequence of a microorganism is not available. The prototype array used comprised 232 plasmids that yielded a reproducible and stable signal. Plasmids contained *M. ulcerans* genomic DNA fragments of 2.3–2.7 kb, thus reaching a theoretical genome coverage of 10%. Despite this incomplete coverage, 12 chromosomal and 3 virulence plasmid–associated RDs were identified. Fifteen distinct deletions of 1.8–53.1 kb were found and characterized in detail by sequence analysis within the 12 genomic RDs. The deletions identified were found in >1 *M. ulcerans* isolate, which demonstrates that they do not reflect events that occur during in vitro cultivation of individual isolates. The diversity of deletions within some genomic regions implies recombination hot spots or a selective advantage for loss of particular sequence stretches. Recombination events between adjacent copies of IS*6110* in *M. tuberculosis* and IS*100* in *Yersinia pestis* have been shown to promote the deletion of intervening DNA segments (*9*,*23*–*26*). Close association of RDs with the high copy number elements IS*2404* and IS*2606* of *M. ulcerans* indicates that these are involved in insertional and deletional events.

Although genome coverage with the prototype microarray used here was low, several geographic types of *M. ulcerans* could be differentiated. The largest group comprised all the African isolates (from Ghana, Benin, Côte d'Ivoire, Democratic Republic of Congo, Angola, and Togo), the isolates from Papua New Guinea, and some of the Australian isolates. A second group comprised the Australian strains 5142 and 5147, and a third group included the South American strains (from Suriname and French Guiana). The Mexican isolates represented a fourth; the Asian isolates (from Japan and China), a fifth subgroup. An extended analysis of insertions and deletions is expected to eventually give insight into the phylogenetic relationship between *M. marinum* and different lineages of *M. ulcerans*. Moreover, the use of a microarray that covers the whole genome

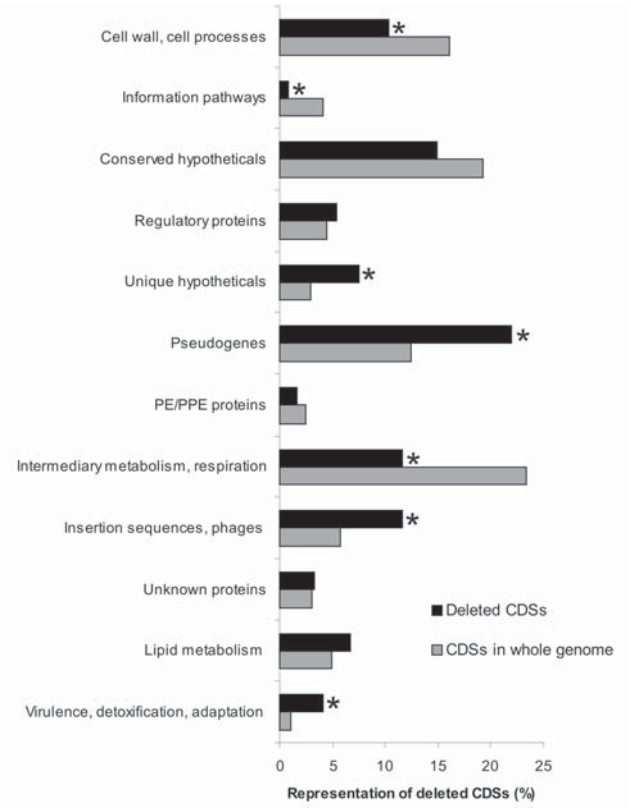

Figure 6. Functional categories of coding sequences (CDSs) and pseudogenes and their frequencies in the deletions and the whole genome. CDSs and pseudogenes in the identified regions of difference (RDs) and in the entire reference *Mycobacterium ulcerans* Agy99 genome were functionally categorized; the frequencies of individual categories are compared. Statistically overrepresented in the RDs were pseudogenes; insertion sequences; CDSs encoding unique hypothetical proteins; and proteins involved in virulence, detoxification, and adaptation. *Significant differences ($p<0.05$).

may lead to the development of a genomic fingerprinting method, which is urgently needed for microepidemiologic studies that aim to characterize transmission pathways and environmental reservoirs of *M. ulcerans*.

The 15 distinct genomic deletions that we identified affected 6.2% of the *M. ulcerans* Agy99 genome, or 5.7% of the annotated CDSs and pseudogenes. When a whole-genome microarray was used to compare genomic DNA of 100 *M. tuberculosis* isolates, 5.5% of the genes were found to be affected (*27*). When one considers the limited genome coverage of the *M. ulcerans* prototype array used here, findings demonstrate a remarkably high degree of insertional and deletional diversity in *M. ulcerans.* In contrast, single nucleotide polymorphisms are rare (*14*).

Comparative genomic studies have shown that *M. ulcerans* recently evolved from the ubiquitous, fast-growing environmental bacterium *M. marinum* (www.sanger.ac.uk/projects/m_marinum) by lateral gene transfer and reductive evolution (*18*). Our comparative genomic hybridization analysis of a worldwide collection of *M. ulcerans* strains indicates that the downsizing of the genome from 6.6 Mb (*M. marinum*) to 5.8 Mb (*M. ulcerans* Agy99) is an ongoing process. Further genome reduction appears to be driving genetic diversification of *M. ulcerans.* Studies of other groups of microorganisms indicate that genome reduction is usually associated with adaptation to a more stable environment. An example is *M. leprae*, which has eliminated >2,000 genes upon adaptation to its human host (*28*). To which ecologic niche(s) in the environment or in host organisms *M. ulcerans* is adapting remains to be investigated.

Among the deleted CDSs are 11 members of the mammalian cell entry *mce3* operon, which are regarded as virulence determinants in other mycobacteria. In *M. tuberculosis* the *mce* operons have been shown to code for genes important for entry and survival of the pathogen in mammalian cells (*29*,*30*). The 4 *mce* operons of *M. tuberculosis* have homologs among other mycobacteria. In particular, the *mce3* operon has been found in *M. avium* and *M. smegmatis;* its deletion in *M. bovis* has been also documented (*31*). The 12.7-kb region that codes for the *mce3* operon is located near the 3′ end of the RD2 element (*32*) that is present in *M. bovis* but absent in some strains of *M. bovis* BCG, which suggests the potential instability of this region. A mouse model of intradermal infection has recently shown that *M. ulcerans* is initially captured by phagocytes (*33*). In vitro studies suggest that the *M. ulcerans* intracellular stage is transient because phagocytic cells enter apoptosis-mediated cell death within 1 day. It will be interesting to investigate whether the *mce3* operon plays a role during the transient invasion of host cells by *M. ulcerans.*

Overrepresentation of proteins involved in detoxification processes among the deleted CDSs indicates adaptation to a more stable environment. Deletion of many dehydrogenases thought to be involved in anaerobic respiration and of anaerobic respiratory enzymes and tranporters may give a hint that this niche is not anaerobic. At least in highly disease-endemic areas, *M. ulcerans'* long-term persistence in chronic wounds and shedding into the environment may be relevant for the propagation of this species. Whether *M. ulcerans* is primarily adapting to persist in a specialized environmental habitat, in arthropod hosts (*34*), or in chronic wounds of mammalian hosts remains to be determined.

Acknowledgments

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Dr Rondini is a microbiologist at the Swiss Tropical Institute, Basel, Switzerland. Her interests focus on the molecular microbiology of *M. ulcerans.*

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Address for correspondence: Gerd Pluschke, Swiss Tropical Institute, Socinstr 57, CH 4002 Basel, Switzerland; email: gerd.pluschke@unibas.ch

Rift Valley Fever Outbreak with East-Central African Virus Lineage in Mauritania, 2003

Ousmane Faye,* Mawlouth Diallo,* Djibril Diop,† O. Elmamy Bezeid,‡ Hampathé Bâ,† Mbayame Niang,* Ibrahima Dia,* Sid Ahmed Ould Mohamed,† Kader Ndiaye,* Diawo Diallo,* Peinda Ogo Ly,* Boubacar Diallo,‡ Pierre Nabeth,* François Simon,* Baïdy Lô,† and Ousmane Madiagne Diop*

In October 2003, 9 human cases of hemorrhagic fever were reported in 3 provinces of Mauritania, West Africa. Test results showed acute Rift Valley fever virus (RVFV) infection, and a field investigation found recent circulation of RVFV with a prevalence rate of 25.5% (25/98) and 4 deaths among the 25 laboratory-confirmed case-patients. Immunoglobulin M against RVFV was found in 46% (25/54) of domestic animals. RVFV was also isolated from the mosquito species *Culex poicilipes*. Genetic comparison of virion segments indicated little variation among the strains isolated. However, phylogenetic studies clearly demonstrated that these strains belonged to the East-Central African lineage for all segments. To our knowledge, this is the first time viruses of this lineage have been observed in an outbreak in West Africa. Whether these strains were introduced or are endemic in West Africa remains to be determined.

Rift Valley fever (RVF) is an acute febrile viral disease that affects small domestic ruminants (*1*) and humans. The disease in animals is characterized by high rates of abortion and death of young ruminants (*2*). In humans, the symptoms are usually mild, but in severe cases, hemorrhages, meningoencephalitis, retinopathy, and sometimes death can occur (*3*). The disease is widespread in Africa, mainly in the sub-Saharan region but also in Egypt. In 2000, outbreaks were recorded for the first time outside of the African continent, in Saudi Arabia and Yemen (*4*). RVF virus (RVFV) belongs to the family *Bunyaviridae*, genus *Phlebovirus* genus, and its genome consists of 3 negative single-stranded RNA segments referred to as L (large), M (medium), and S (small) (*5*).

In West Africa, the first extensive RVF outbreak recorded to date occurred in Mauritania in 1987 and resulted in 220 human deaths (*6*). After this outbreak, an active surveillance system led to the detection of several animal cases in Mauritania, Senegal, and other West African countries (*7*–*9*). Furthermore, during interepizootic periods, RVFV has been repeatedly isolated from different mosquito species in Senegal, Burkina Faso, and Nigeria (*10*–*12*,). During 1998, an outbreak of RVF occurred in southeastern Mauritania, resulting in 300 to 400 human cases and 6 deaths (*13*).

In Mauritania in 2000, health authorities established a National Disease Surveillance System (NDSS) by using sentinel herds in 5 geographic regions and a notification system of hemorrhagic fever in medical healthcare centers. This NDSS was implemented in collaboration with the Centre National d'Hygiène, the "Centre National d'Elevage et de Recherche Vétérinaire" in Mauritania, and the Institut Pasteur de Dakar in Senegal. The value of the NDSS was further reinforced after an outbreak of Crimean-Congo hemorrhagic fever in Mauritania in February 2003 (*14*) for which RVFV was identified in animal and human serum specimens collected in September and October 2003. We describe the results of a multidisciplinary investigation to determine extent of the outbreak and the key factors responsible for RVFV reemergence in Mauritania.

Materials and Methods

Case Definitions

A suspected human RVFV case-patient was defined as a person with fever associated or not with hemorrhagic,

*Institut Pasteur de Dakar, Dakar, Sénégal; †Institut National de Recherche en Santé Publique, Nouakchott, Mauritania; and ‡Centre National d'Elevage et de Recherche Vétérinaire, Nouakchott, Mauritania

jaundice, or neurologic symptoms or any person who died who had had overt hemorrhagic fever symptoms from September through December 2003. A confirmed human RVFV case-patient was defined as a person for whom laboratory tests confirmed an acute or recent RVFV infection, e.g., by ≥1 of these results: immunoglobulin M (IgM), reverse transcription–PCR (RT-PCR) or virus isolation positive results. A human contact was defined as any person or relative who had been directly in contact with a confirmed human or animal case-patient, or with a person who died who had had overt hemorrhagic fever symptoms from September through December 2003.

Field Investigations

Study Sites

Nine localities belonging to 3 administrative provinces were visited (Figure 1): Keur Macène and Rkiz (Trarza Province), Makhtar Lahjar, Guimi, Taïba, and Sagle Moure (Brakna Province), Legrane, Kélébélé, and Hseytine (Assaba Province). These localities were chosen because they had confirmed human or animal cases.

Human Investigations

In affected areas, the investigation was conducted under the supervision of the chief of the sanitary district. For each case, venous or capillary blood samples were collected into dry tubes or onto filter papers, respectively. A thick blood smear was also taken from all suspected case-patients for differential diagnosis of malaria. An interview in which information was gathered about sex, age, date of fever onset, and hemorrhagic signs was conducted for all case-patients and their contacts.

Animal Investigations

All domestic animals living in the close vicinity of suspected or confirmed case-patients were included in the study. Every blood sample was accompanied by an investigation form specifying the species, age, and localization of the animal during the month before the investigation and, for female animals, a history of pregnancies.

Entomologic Investigations

Adult mosquitoes were collected in CDC light-traps (*15*), with or without CO_2, which were placed close to water points or in sheepfolds, respectively; animal-baited traps were placed in the houses of persons with suspected or confirmed cases. Mosquitoes were frozen and subsequently identified on a chilled table by using morphologic keys (*16*,*17*). They were classified into monospecific pools, stored in liquid nitrogen, and transported to the laboratory, where they were kept at –80°C until virus isolation was attempted.

Figure 1. Locations of the study sites.

Laboratory Tests

Serologic Studies

All human and animal samples were tested for evidence of IgG and IgM by using an ELISA technique (*18*,*19*). Serum specimens were considered positive for antibodies if the difference between the sample and control optical densities was >3 standard deviations above the mean of the negative controls.

Molecular Studies

Viral RNA was extracted from serum of suspected case-patients by using the QIAamp RNA kit (QIAGEN, Inc. Chatsworth, CA, USA) and RT-PCR was done by using the Titan One-Step RT-PCR System (Roche Diagnostics, Mannheim, Germany), according to the recommendations of the manufacturers. The primers NS3a (nt 710–729; 5′-ATGCTGGGAAGTGATGAGCG-3′) and NS2g (nt 61–80; 5′-TGATTTGCAGAGTGGTCGTC-3′) were used to amplify a 669-nt region of the virus S segment region encoding the NSs protein. The primers MRV1a (nt 772–790; 5′-CAAATGACTACCAGTCAGC-3′) and MRV2g (nt 1563–1580; 5′-GGTGGAAGGACTCTGCGA-3′) were used to amplify a 809-nt region of the virus M segment region encoding the G2 protein. Primers Wag (nt 4440–4457; 5′-ATTCTTATTCCCGAATAT-3′) and Xg (nt 4634–4651; 5′-TTGTTTTGCCTATCCTAC-3′) were used to amplify a 212-nt region of the L segment (*20*–*22*). The PCR products were purified on agarose gel and directly sequenced by using the Sanger method with an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA). Phylogenetic trees on the partial sequences of the S (601 nt), M (726 nt), and L (121 nt) RNA segments were constructed by using the maximum likelihood method (PAUP* 4.0, Sinauer Associates Inc., Sunderland, MA, USA).

Virologic Studies

Virus isolation was performed at the World Health Organization (WHO) Collaborating Center for Arbovi-

ruses (www.pasteur.fr/recherche/banques/crora), Institut Pasteur de Dakar on mosquitoes and serum collected from humans by inoculating the virus into suckling mice and a mosquito cell line (AP61). Virus identification was performed by an indirect immunofluorescence assay that used polyclonal and monoclonal antibodies. The identification of virus isolates was confirmed by complement fixation (*23*).

Parasitologic Test

To rule out malaria infection, thick blood smears from patients with suspected cases were Giemsa-stained. The ratio of parasite (*Plasmodium falciparum*) to leukocytes was estimated in 200 fields based on a mean leukocyte count of 8,000/μL of blood.

Results

Human Cases

The different cases recorded and the linkages between them are represented in Figure 2. The 9 confirmed case-patients, identified before the investigation, were from the Assaba, Brakna, Trarza, and Tagant provinces. Subsequent discussions with the head of the sanitary district enabled the localization of the residences and the relatives for 2 of them (index case-patients 1 and 2). Several confirmed cases-patients (index case-patients 3 to 9) and their relatives were not found due to the great distances between localities or nomadic behaviors of populations. However, for all suspected case-patients (S), further investigation was conducted in the provinces where the confirmed case-patients lived.

In total, 98 persons (66 contacts, 23 with suspected cases, and 9 with confirmed cases) were included in this study. Of these persons, 25.5% (25/98) had evidence of recent RVFV infection (i.e., presence of IgM, viral RNA or virus, or >1 of these results), and 10% had evidence of past infection (i.e., presence of IgG alone). Seven viral strains were isolated. For the 25 patients who were recently infected (9 with confirmed cases before the investigation, 6 contacts, and 10 with suspected cases at the time of investigation), the median age was 21 years (range 7–50 years), 4 died, and 16 had hemorrhagic signs (hematemesis, vaginal bleeding, severe hemoptysis, bleeding from the gums and venipuncture sites, petechial rashes, and ecchymoses of the skin). Among the 23 suspected case-patients from whom blood samples were collected, 10 were infected by RVFV; only 2 patients were positive for malaria parasites.

In Assaba Province, 2 confirmed case-patients were recorded before the investigation (index case-patients 1 and 2), and 1 suspected case-patient (S1) was found during the investigation. The index case-patient 1 was dead at the time of the investigation; however, RVFV (strain SHM169867)

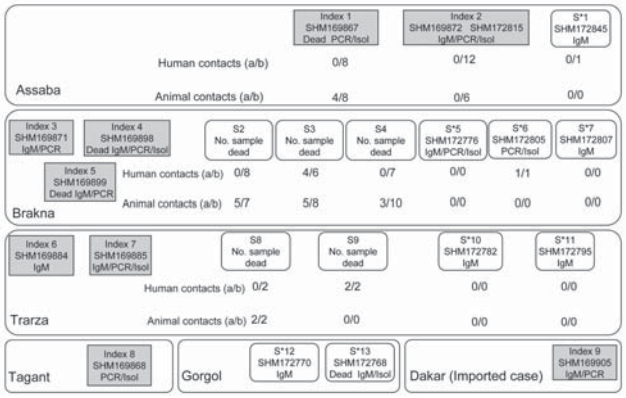

Figure 2. Investigation of human and animal contact around index and suspected case-patients (S) from Mauritania in 2003. For each case-patient (represented as a box), PCR, immunoglobulin M (IgM), or isolation (Isol)-positive test results are indicated below the sample number (e.g., 169867). (a/b), no. IgM positive/no. tested; S*, suspected case-patient before field investigation and subsequently confirmed positive by laboratory tests.

was isolated from a blood sample taken during his illness. No RVFV infection was noticed in contact persons associated with this patient. However, IgM against RVFV was detected in animals living in and near the residence of index case-patient 1. For index case-patient 2 (infected with the strain SHM169872), a second blood sample was taken, and presence of IgM antibodies against RVFV was confirmed. No evidence of RVFV infection was detected in contact persons or in animals living in or near the residence of this patient. The suspected case-patient 1 was identified in Kiffa Hospital, where he was admitted on November 3, 2003. This 50-year-old patient had onset of fever on October 24, 2003, with asthenia, jaundice, nausea, hematemesis, epistaxis, and gingival hemorrhage. Serologic tests showed IgM against RVFV. No evidence of virus infection was detected in those who had accompanied this patient to the hospital.

In Brakna Province, where 3 cases were confirmed before the investigation (index case-patients 3–5), 6 suspected case-patients (S2–S7) were found during the investigation. Laboratory testing of samples from the 3 index case-patients showed IgM against RVFV by ELISA and RVF viral RNA by RT-PCR. A virus strain (SHM169898) was isolated from index case-patient 4. Among the suspected case-patients from this province, 3 deaths (S2–S4) were recorded, but no samples were available from those patients. Nevertheless, animals in the vicinity of S1, S2, and S3, were found to be infected (5/7, 5/8, and 3/10 animals, respectively). In addition, infection was detected in 4 of 6 persons who had been in contact with suspected case-patient 3. In contrast, no evidence of infection by RVFV was detected in contacts of S2 and S4. The suspected case-

patient 5 was a student living in Makhtar Lahjar, who had fever onset on October 20, 2003, was admitted to the National Hospital Center (NHC) of Nouakchott on November 6, and from whose blood RVFV (strain SHM172776) was subsequently isolated. During the investigation, 2 suspected case-patients (S6 and S7) were discovered in the Healthcare Center of Makhtar Lahjar. Suspected case-patient 6 was a 17-year-old female patient who had onset of fever on October 10 and who was admitted to the center on November 1 with headache, abdominal pain, vomiting, hemorrhages, and epistaxis; virus was isolated from this patient (strain SHM172805). The contacts associated with suspected case-patient 6 were also found to be infected with RVFV. Suspected case-patient 7 was a 35-year-old man. He had a fever on October 15 and was admitted to the Health Center of Makhtar Lahjar on November 1 with headache, nausea, vomiting, and epistaxis. The RVF IgM test result for this patient was positive for RVFV.

In Trarza Province, 2 confirmed case-patients were observed during the period of surveillance (index case-patients 6 and 7) and 4 suspected case-patients (S8–S11) were identified during the investigation. Viral isolation was positive for the index case-patient 7 (strain SHM169885). Among the suspected case-patients, 2 (S8 and S9) died before blood samples could be obtained. However, blood testing of samples from 2 animals living in the vicinity of S8 and from 2 human contacts of S9 found recent RVFV infection. Suspected case-patient 10 was 28-year-old man, with onset of fever on October 22, who came for consultation to Keur Macene Healthcare Center. He had a prolonged cough without hemorrhagic symptoms, and an IgM ELISA result for RVFV was positive. The suspected case-patient 11 is a 26 year-old woman, with onset of fever on October 22, who was admitted to the healthcare center of Rkiz with headaches, asthenia and anorexia without hemorrhagic signs. The RVF IgM test result of this patient was positive. In Tagant Province, index case-patient 8, whose condition was diagnosed before the investigation, was a man who came for consultation at the provincial hospital on September 24, exhibiting fever and hematemesis. RVFV (strain SHM169868) was isolated from a blood sample taken on September 29.

In Gorgol Province, 2 suspected case-patients (S12 and S13) were evacuated to the NHC of Nouakchott. The onset of their symptoms dated to October 23 and October 25, respectively. Samples from each were positive for IgM against RVFV by October 30, and RVFV (strain SHM172768) was isolated from S13, who died on October 30.

In Dakar, Senegal, an "imported" case (index case-patient 9) in a person from Rosso, Mauritania, was diagnosed by positive results by ELISA IgM and RT-PCR. This patient was first admitted to the NHC of Nouakchott, Mauritania, before being transferred to the Hôpital Principal de Dakar.

Animal Cases

Serum samples were obtained and tested from 54 domestic animals (48 goats and 6 sheep) living in the visited localities (Table 1). The median age was 4 years (range 1–15), and the abortion rate was 70% during the last gestation period. IgM against RVFV was detected by ELISA in 25 of 54 animals; no IgG against RVFV was found in any of the 54. Among the animals with a positive test result, the abortion rate was 92%.

Mosquitoes

A total of 22,201 mosquitoes, belonging to 4 genera and 17 species, were collected. *Culex poicilipes* was the most frequent species (43.8%), followed by *Cx. antennatus* (23%) and *Mansonia uniformis* (9%). A total of 544

Table 1. Prevalence rate of immunoglobulin M against Rift Valley fever virus in livestock from Mauritania, 2003

District/Locality	Month sampled	Livestock species (no. positive/no. tested)			No. abortions/ no. tested
		Sheep	Goats	Total	
Brakna					
Taiba	Oct	0/1	3/9	3/10	6/10
Guimi	Oct	1/1	4/7	5/8	8/8
Sagle Moure	Nov	0/0	5/7	5/7	5/7
				13/25 (42%)	19/25 (76%)
Trarza					
Boynayé	Oct	0/3	0/6	0/9	0/9
Rkiz	Oct	0/0	2/2	2/2	2/2
				2/11 (18%)	2/11 (18%)
Assaba					
Legrane	Nov	0/1	2/5	2/6	5/6
Kélébélé	Nov	0/0	1/2	1/2	2/2
Tézékré	Nov	0/0	1/2	1/2	2/2
Hseytine	Nov	0/0	6/8	6/8	8/8
				10/18 (55%)	17/18 (94%)
Total		1/6 (16%)	24/48 (50%)	25/54 (39%)	38/54 (70%)

monospecific pools were constituted and submitted for viral isolation. Only *Cx. poicilipes* was found to be associated with RVFV. Three strains (ArD 174367, ArD 174303, and ArD 174347) were isolated from the 146 pools constituted in Guimi Province, giving rise to a minimum infection rate of 0.04% for this locality and 0.01% for the whole study site (Table 2).

Genetic Analysis

RNA was extracted from the 8 viral strains isolated from humans, and fragments of the S, M, and L segments were amplified and sequenced. No amino acid (aa) differences were found between the fragments of the S or L segments analyzed (198 and 51 aa, respectively). A single amino acid difference was found between the M fragment (255 aa) of the 5 viral strains analyzed. Results of phylogenetic analyses of the nucleotide sequences of amplified fragments from 3 segments belonging to 2 representative strains (H1MAU03 [SHM169867] and H2MAU03 [SHM169868]) isolated during this epidemic and previously described nucleotide sequences of RVFV are shown in Figure 3. The strains identified in Mauritania 2003 are consistently located within the East/Central lineage for all trees. This lineage contains viral strains that circulated in Madagascar (1991), Kenya (1997), Chad (2001), and Saudi Arabia (2001).

Discussion

The combination of ELISA, RT-PCR, and isolation assays has permitted the rapid and efficient identification of RVFV as the cause of the extended hemorrhagic fever outbreak reported in Mauritania during the last quarter of 2003. Of the 24 RVF cases diagnosed in the laboratory, 13 were diagnosed by IgM only; 8 were diagnosed by IgM, RT-PCR, isolation, or >1 method; and 3 were diagnosed by RT-PCR, isolation, or both. These data and those obtained during the epidemics of RVF in Kenya (*25*), as well as in Saudi Arabia and Yemen (*4*), demonstrate the importance of combining diagnostic assays for accurate and comprehensive detection of RVFV infection.

Regarding differential diagnosis, only 2 suspected case-patients with fever had confirmed malaria due to infection by *P. falciparum*. This low malaria infection rate suggests that the RVF outbreak was the major cause of the febrile cases notified during this period.

Although WHO estimates that the human mortality rate due to RVFV is ≈1%–2% of infected patients, the number of recorded deaths during this outbreak was 4 among 25 infected patients when the laboratory data were considered exclusively. Epidemiologic investigations have found 5 additional deaths that could be due to RVFV infection. We cannot be absolutely certain about the causes of death in our suspected case-patients from whom no blood sample was taken. However, when the clinical symptoms and the rate of infection in domestic animals are considered, that these cases were the result of RVFV infection is highly probable. In those cases in which the contacts had negative test results and only domestic animals had positive results, we hypothesize that the infection of those with lethal cases was related to socioeconomic/professional activity. Indeed, those at highest risk include butchers and others who come in contact with animals (e.g., slaughterhouse workers, tanners, and herdsmen), who represent a large part of the population living in these areas.

During this investigation, a high infection rate was found in sheep and goats that lived in close proximity to the patients (46.3% of IgM positive compared with 25% during the 1998 outbreak) (*13*). Also, according to interviews with herdsmen, a high abortion rate (92%) was observed in infected animals during their most recent pregnancy. Previous studies showed abortion rates ranging from 80% to 100% (*26*). Nevertheless, the discrepancies

Table 2. Mosquitoes collected during the Rift Valley fever outbreak, Mauritania, 2003

	Culex antennatus		*Cx. poicilipes*		*Cx. tritaeniorhynchus*		*Mansomia uniformis*		Others*		Total	
Locality	C	P	C	P	C	P	C	P	C	P	C	P
K. Macene	273	8	420	12	195	5	1,755	67	365	18	3,008	110
Boynayé	46	1	137	4	766	17	159	5	667	14	1,775	41
Techtayatt	696	14	290	7	451	9	30	1	627	15	2,094	46
Aleg	1,694	35	1437	30	155	3	69	2	435	12	3,790	82
Taiba	2,243	45	255	7	38	2			540	17	3,076	71
Boghe									16	3	16	3
Guimi	159	3	7163	146†					943	21	8,265	170
Legrane			6	1					14	2	20	3
Kélébélé			11	2					23	4	34	6
Hseytine									8	3	8	3
Sarandougu	8	1	18	1	3	1			86	6	115	9
Total	5,119	107	9,737	210	1,608	37	2,013	75	3,724	87	22,201	544

**Aedes. vexans*, *Ae. ochraceus*, *Anopheles funestus*, *An. gambiae*, *An. pharoensis*, *An. rufipes*, *An. wellcomei*, *An. ziemanni*, *An. squamosus*, *Cx. ethiopicus*,*Cx. neavei* and *Ma. African*. C, collected; P, pools.
†Rift Valley fever virus strains (ArD 174367, ArD 174303, and ArD 174347) isolated in 3 pools.

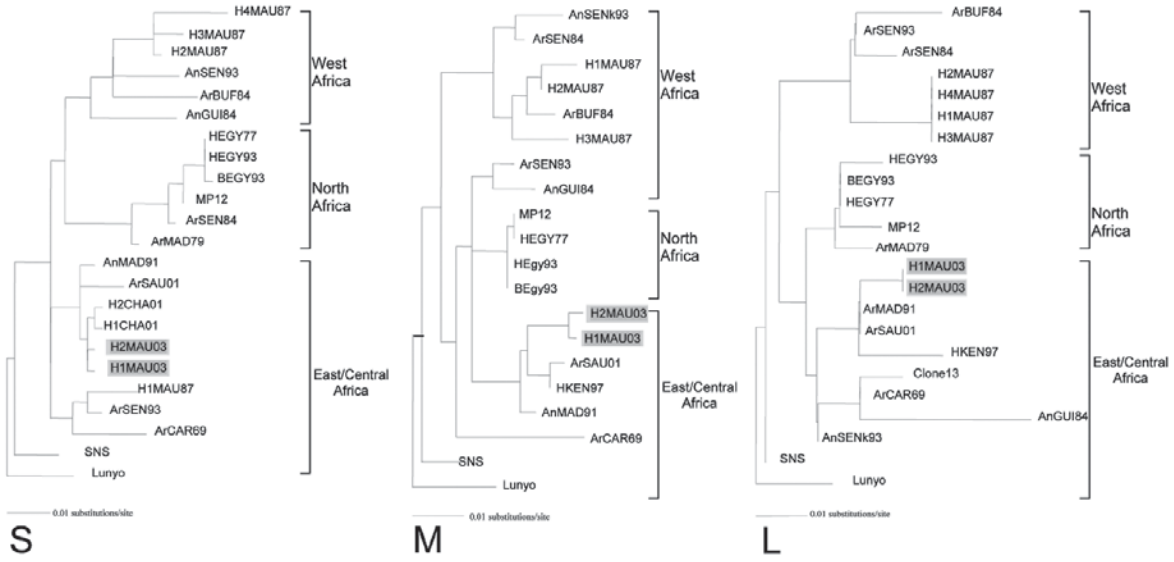

Figure 3. Phylogenetic relationships of the S (small), M (medium), and L (large) RNA segments of Rift Valley fever viruses. Strains isolated in Mauritania (gray shading) are designated H1MAU03 and H2MAU03, according to previous abbreviation guidelines (*24*). Nucleotide sequences of these segments (S, M, and L) have been submitted to GenBank with the following accession nos., respectively: EF160113, EF160116, and EF160117 for H1MAU03; EF160114, EF160115, and EF160118 for H2MAU03. Branch lengths are proportional to the number of substitutions per site.

observed between the overall abortion rate in animals and the prevalence of anti-RVF IgM (70% vs 46.3%) support the hypothesis of the existence of other cocirculating diseases that also cause abortion. The lack of anti-RVF IgG in domestic animals is surprising considering the long-standing virus endemicity in Mauritania. This observation could be ascribed to different factors: 1) the small number of domestic animals analyzed, 2) a recent introduction of the virus in these localities, or 3) a renewal of animal populations (*27*). The latter hypothesis is supported by the observation of the relatively young median age of the animals tested (4 years old), reflecting a new animal population since the 1998 RVF epidemic in Mauritania.

Among the mosquitoes collected, several species known as RVFV vectors (*Cx. poicilipes, Cx. tritaeniorhynchus, Cx. antennatus*, *An. pharoensis*, *Ae. vexans*, *Ae. ochraceus*, and *Ma. africana*) (*10*,*28*) were recorded, but only *Cx. poicilipes* was found to carry RVFV during the outbreak. Spatial analyses of the results show that *Cx. poicilipes* was in fact predominant only in the village of Guimi, where the RVFV was isolated. This observation indicates that the levels of the different species vary according to the local environment. Mosquitoes from the *Aedes* genus, known for their role in RVFV maintenance and transmission, were scarce, likely due to their early appearance at the onset of the rainy season, whereas our investigation took place at the end of the rainy season. Indeed, in 2003, the last rainfall event was recorded at the beginning of October.

Genetic analyses of the 3 segments of RVFV isolated during this epidemic showed a low level of variation between isolates from the different provinces. This finding supports the hypothesis that the same strain was circulating in the different affected areas. The nucleotide sequences of the strains isolated during this epidemic compared with those isolated elsewhere in Africa and Saudi Arabia showed that they belong to the East/Central African cluster for the 3 segments. Previous reports have shown that some strains isolated in West Africa share 1 or 2 segments with strains belonging to the East/Central African cluster (*2*,*29*). However, to our knowledge, this is the first evidence of the circulation in West Africa of strains harboring 3 segments that all belong to the East/Central African cluster.

This finding confirms the existence of RVFV strain exchanges between geographic areas. In fact, the spread of RVFV from East Africa to other regions has already been observed during the RVF outbreak in Saudi Arabia and Yemen in 2000–2001 (*4*) and in Chad in 2001 (*30*). RVFV was also found to be the cause of the epidemic/epizootic in Egypt in 1977 and in Madagascar in 1979 (*31*). Such a mechanism of RVFV spread likely depends on human and animal population movements for which animal migration routes between West and East/Central Africa need to be identified. Furthermore, previous studies have demonstrated that reassortant viruses can emerge when 2 RVFV lineages coexist (*24*). Favorable environmental conditions (mainly the rainfall pattern), which led to the emergence of already introduced East/Central African strains, seem to

be the cause of this outbreak. Indeed, RVFV emergence in East Africa is undoubtedly the consequence of rainfall surplus (*32*,*33*). In contrast, in West Africa, the few studies carried out in the past indicate that rainfall surplus is not a key factor for RVFV emergence. In fact, RVF outbreaks were often observed during years of rainfall deficit (*10*,*34*). Therefore, this outbreak in Mauritania, caused by RVFV strains of the East/Central African lineage, is likely linked to the heavy rainfall recorded during 2003 in the affected areas (315 mm in 2003 versus 161 mm in 2002). These arguments support the hypothesis that episodes of heavy rainfalls are directly or indirectly more favorable to the emergence of virus strains belonging to the East/Central African cluster. However, such a hypothesis presumes the existence of ecologic or biologic differences between strains of the 2 lineages, and further investigations are needed with special emphasis on the interactions with the strains' respective vectors and reservoirs.

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Dr Faye is a virologist and acarology specialist who works at the Department of Virology, Institut Pasteur de Dakar, Senegal. His primary research interests include the virology and vectorial transmission of arboviruses and viral hemorrhagic fevers.

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Address for correspondence: Ousmane Madiagne Diop, Département de Virologie, Institut Pasteur de Dakar, 36 Ave Pasteur, Boîte Postale 220, Dakar, Sénégal; email: diop@pasteur.sn

Response to Emerging Infection Leading to Outbreak of Linezolid-Resistant Enterococci

Marion A. Kainer,* Rose A. Devasia,*† Timothy F. Jones,* Bryan P. Simmons,‡ Kelley Melton,‡ Susan Chow,‡ Joyce Broyles,‡ Kelly L. Moore,* Allen S. Craig,* and William Schaffner§

Linezolid was approved in 2000 for treatment of gram-positive coccal infections. We performed a case–control study during a hospital outbreak of linezolid-resistant enterococci (LRE) infections, comparing cases of LRE infection (cases) with linezolid-sensitive enterococci infections (controls). Nasal and perirectal swab samples were obtained from all patients in a 1-day point-prevalence survey. We examined antimicrobial drug use and calculated the defined daily dose of linezolid per 1,000 patient-days. Fifteen LRE cases were identified (13 *Enterococcus faecalis* and 2 *E. faecium*); 7 were vancomycin-resistant. Compared with controls, case-patients had increased in-hospital mortality rates and lengths of stay. Multivariate analysis identified independent predictors of LRE infection: prior cultures positive for methicillin-resistant *Staphylococcus aureus* (adjusted odds ratio [AOR] 27), hospitalization duration before index culture (AOR 1.1 per day), and duration of preceding linezolid therapy (AOR 1.1 per day). Linezolid exposure and patient-to-patient transmission appear to be responsible for LRE infections, an important emerging hospital problem.

Enterococci are common inhabitants of the human gastrointestinal tract. Although >40 enterococcus species exist, nosocomial infections are primarily caused by *Enterococcus faecalis* and *E. faecium* (*1*). Enterococcal infections are the third most common cause of nosocomial infection in intensive care units (ICUs), and multidrug-resistant enterococcal infections have been associated with higher hospitalization costs and a higher number of related deaths (*2*,*3*).

*Tennessee Department of Health, Nashville, Tennessee, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡Methodist University Hospital, Memphis, Tennessee, USA; and §Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Linezolid, 1 of the oxazolidinone class of antimicrobial drugs, inhibits bacterial protein synthesis by binding to the 50S subunit of 23S rRNA. In April 2000, linezolid was approved in the United States and has been heavily marketed to treat methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) infections (*4*,*5*). Although more expensive than vancomycin, linezolid does not require testing for adequate serum drug concentrations or dosing adjustment for renal or hepatic insufficiency (*6*), and it has been regarded by some healthcare providers as more effective than vancomycin in treating nosocomial pneumonia and MRSA skin and soft tissue infections (*7*–*9*). Most reports of linezolid-resistant enterococci (LRE) have been individual cases or small case series (*10*–*20*) or have specifically described linezolid-resistant and vancomycin-resistant *E. faecium* (LRVRE) (*17*–*22*). We describe a large hospital outbreak of LRE infections.

Hospital A is an urban, 500-bed, adult inpatient, teaching facility with surgical, transplant, and medical ICUs in a city of ≈850,000 persons. Community-associated MRSA is an important emerging pathogen in that city (*23*,*24*). At hospital A in 2004, 154 MRSA and 29 VRE blood culture isolates were identified; compared with results for 1997, these are increases of 428% and 725%, respectively (Figure 1). Linezolid became available in hospital A in April 2000, but it was restricted for use by infectious disease and critical care physicians only, some of whom believed it provided an advantage over vancomycin for treatment of MRSA, especially pulmonary MRSA (*8*,*9*). In February 2005, hospital A's infection-control staff contacted the Tennessee Department of Health after isolating LRE in the blood culture of a ventilated patient. Within a week, surveillance cultures identified a second patient with LRE in the same unit. We undertook an investigation to characterize the epidemiology of LRE, to determine risk factors

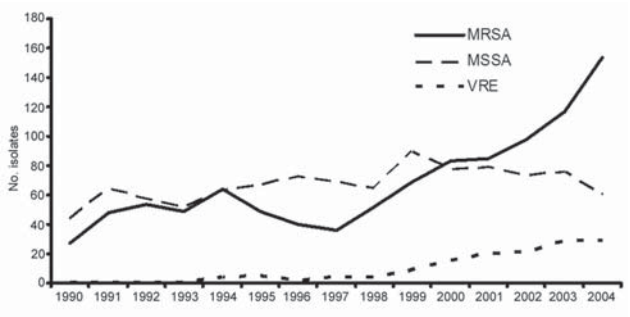

Figure 1. No. nonduplicate blood-culture isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), and vancomycin-resistant *Enterococcus faecalis* and *E. faecium* (VRE) per year, hospital A, Tennessee, 1990–2004.

for emergence of linezolid resistance in enterococci among patients previously infected or colonized with linezolid-sensitive enterococci (LSE), and to determine outcomes associated with LRE infection. This investigation was approved by the institutional review board of hospital A.

Methods

Epidemiology

Cases were identified by manually reviewing hospital A's microbiology susceptibility testing reports related to all *E. faecium* and *E. faecalis* isolates for January 2004 through February 2005. Patients for whom a clinical isolate of LRE had been identified during the study period were selected for the study. For each case, 4 randomly selected hospitalized control subjects with LSE were identified by using hospital microbiology reports; no matching was performed. The index hospitalization was defined as the hospital admission during which LRE or LSE had been identified. Trained staff performed chart reviews by using standard questionnaires to determine demographics, hospital course, immunocompromising conditions, instrumentation during the index hospitalization, and history of hospitalization and inpatient antimicrobial drug exposure during the 12 months preceding the index isolate. Instrumentation was defined as receipt of Foley catheterization, chest tube, Swan-Ganz catheterization, mechanical ventilation, dialysis catheter, central line, arterial line, peripherally inserted central catheter, permanent central venous catheter, balloon pump, or intraabdominal or other surgery. Immunocompromising conditions were defined as the presence of leukemia or nonskin cancer, chronic renal failure, requiring dialysis, diabetes, HIV infection, pancreatitis, steroid use of ≥10 mg for >5 days, or solid organ or stem cell transplantation. Mortality was defined as patient death during the index hospitalization. Critical care areas were defined as the ICUs, extended postoperative holding area (overflow ICU), coronary care unit, and ventilator rehabilitation unit.

Specimen information for clinical isolates of *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis* was obtained for the index hospitalization and for the preceding 12 months. Isolates from surveillance cultures (not illness-associated) were not included. Invasive clinical isolates were defined as isolates from any of the following sources: blood; bone; cerebrospinal, joint, pericardial, peritoneal, or pleural fluid; surgical specimen or aspirate; or any other normally sterile site. Noninvasive isolates included isolates from sputum, urine, or wounds. We performed a subset analysis comparing linezolid-sensitive and vancomycin-resistant enterococci (LSVRE) and LRVRE infections (case–control study II). After the initial random selection of controls with LSE infections, we selected additional controls with LSVRE to obtain a 1:4 ratio of LRVRE-infected case-patients to LSVRE controls.

Point-Prevalence Survey

A 1-day point-prevalence culture survey for *E. faecalis, E. faecium*, and *S. aureus* was performed in hospital A. On March 28, 2005, all patients hospitalized in hospital A were asked if they would give informed consent for the collection and culture of nasal and perirectal swab specimens; specimens were obtained from all patients who gave consent. Identification and antimicrobial drug susceptibilities of nasal and perirectal swab specimen cultures were performed in hospital A's microbiology laboratory.

Laboratory Studies

Hospital A used the Dade Microscan Walkaway 96 (Diamond Diagnostics, Holliston, MA, USA) SI Pos Combo 21 for all species identification and susceptibility testing of gram-positive clinical isolates (*25*). Linezolid resistance was confirmed at the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) by use of the broth-dilution method; the MIC was ≥16 μg/mL for all isolates tested. For the point-prevalence survey, hospital A microbiology staff plated nasal swabs specimens onto mannitol-salt agar plates; linezolid (30 μg) and oxacillin (1 μg) disks were placed in the first quadrant to screen for LRSA and MRSA, respectively. Perirectal swabs samples were plated directly onto 2 bile-esculin plates, 1 of which contained 6 μg vancomycin/mL to screen for VRE. A linezolid disk was placed on the heavy inoculum on the plate without vancomycin to screen for LRE.

Pulsed-field gel electrophoresis (PFGE) subtyping was performed at the Tennessee Department of Health laboratory on available LRE isolates (clinical [3], surveillance [3], and environmental [3]) from hospital A. The PulseNet (CDC) standardized protocol for *Listeria monocytogenes* (*26*) was used for DNA preparation. Specific conditions

adapted for this application included separate 5-h digestions with 100 U *Sma*I and 100 U *Apa*I restriction endonucleases and 18-h electrophoresis, using a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA, USA) programmed for a molecular weight range of 25–350 kb, an initial switch time of 2.0 s, and a final switch time of 20.0 s. The PulseNet size standard, *S.* Braenderup strain H9812 (ATCC BAA-664) (*27*), was used as a size marker. PFGE fingerprint types were assigned by using the criteria of Tenover et al. (*28*).

By using laboratory information system data, we determined the number of MRSA, methicillin-sensitive *S. aureus*, and VRE isolates cultured from hospital A inpatient blood samples from January 1990 through December 2004. Repeat isolates from the same patient within 30 days were excluded.

Antimicrobial Drug Usage

The defined daily dose (DDD) of linezolid was identified as 1.2 g/day. We reviewed hospital A's pharmacy purchase data for linezolid for October 2001 through February 2005 and calculated DDD/1,000 patient-days; intravenous linezolid usage was analyzed by patient location (ICU or non-ICU). Oral linezolid use was not tracked by patient location. Hospital A's antimicrobial drug–prescribing restrictions were reviewed. Hospital A pharmacy staff conducted a drug-usage evaluation of linezolid for January through April 2005. The drug-usage evaluation included prescriber information, duration, and indication for linezolid; patients with active linezolid orders were identified by concurrent computer printouts.

Statistical Analyses

Statistical analyses were performed by using Epi Info 3.2.2 (CDC) and SAS v. 9.1 software (SAS Institute, Cary, NC, USA). Fisher exact test was used to compare categorical variables; the Kruskal-Wallis test was used to compare continuous variables. For univariate analysis, exact methods were used for 95% confidence intervals (CIs); for multivariate analysis, 95% Wald CIs were used. All p values were 2-sided.

Results

For January 2004 through February 2005, a total of 15 LRE cases were identified (Figure 2): 2 (13%) were *E. faecium* and 13 were *E. faecalis* infections. Of the 15 case-patients, 12 (80%) were black and 8 (53%) were female; the median age was 54 years (range 38–74 years) (online Appendix Table, available from www.cdc.gov/EID/content/13/1024-appT.htm). Two of the case-patients had been admitted to the hospital from a nursing home. For 8 of the 15 patients, diabetes was a previous medical condition; 2 patients had previously required dialysis, and no patients had been transplant recipients. Eight patients had required care in a critical-care area before acquiring an LRE infection. Six (40%) case-patients died.

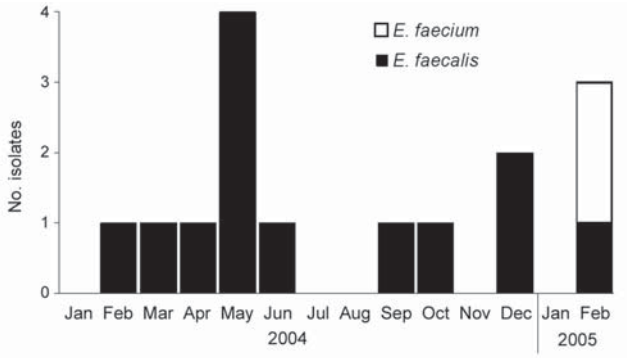

Figure 2. Occurrence of linezolid-resistant *Enterococcus faecalis* and *E. faecium* in hospital A, Tennessee, January 2004–February 2005 (N = 15).

Case–Control Study I

Sixty control patients with LSE clinical isolates were identified. Case-patients and LSE patients did not differ significantly by age, race, sex, immunocompromising conditions, or place of origin (e.g., nursing home) (Appendix Table). Of the enterococcal isolates from case-patients and controls, 13 (87%) and 53 (88%), respectively, were *E. faecalis*. LRE isolates were more likely to have been invasive than LSE isolates (8 [53%] vs. 12 [20%]; odds ratio [OR] 4.6; 95% CI 1.2–17.9).

Patients with LRE infection were more likely to die during their hospitalization than those with LSE infection (OR 9.3; 95% CI 1.8–51.2). Case-patients were hospitalized significantly longer than controls (median 35 days [range 1–127] for case-patients vs. 11 days [range 1–140] for controls; $p<0.001$). Case-patients were hospitalized for longer periods than control patients before the index culture (20 vs. 4 days; $p<0.001$) and after the index culture (19 vs. 9 days; $p = 0.002$).

During the preceding 12 months, LRE case-patients had received more cumulative days of antimicrobial drug treatment than LSE patients (median 58 vs. 18 days; $p = 0.003$). Compared with patients with LSE infection, patients with LRE infection had more frequently received linezolid therapy during the prior 14 days (27% vs. 5%; OR 9.7; 95% CI 1.3–76.8). This association remained valid for receipt of linezolid in the prior 2, 3, and 12 months. Patients with LRE infection, compared with patients with LSE infection, also had more cumulative days of linezolid therapy (median 15 vs. 0 days; $p = 0.009$); among those who had received linezolid, the median was 17.5 days versus 8 days ($p = 0.06$). Case-patients were less likely than control patients to have received vancomycin during the previous year. Apart from carbapenems, no other antimicrobial drugs were associated

with LRE infection; carbapenems were usually prescribed together with linezolid.

Case-patients with LRE were also more likely than controls to have had a prior clinical isolation of MRSA (OR 13; 95% CI 3.0–60.4): 10 (67%) case-patients and 8 (13%) control patients had an MRSA infection during the 12 months before the index hospitalization. Of the 10 case-patients with a previous MRSA infection, 5 had received linezolid therapy (4 after isolation of MRSA) before their LRE infection.

Next, we stratified prior infection with MRSA by linezolid exposure. Among patients who had no linezolid exposure, prior MRSA infection remained strongly associated with subsequent LRE infection (OR 23.0; 95% CI 2.6–272.0). Conversely, when we stratified linezolid exposure by prior MRSA infection, the exposure was associated with LRE infection among those with no previous MRSA infection (OR 11.5; 95% CI 1.0–152.8). The association between prior MRSA infection and hospital stay before the index culture did not reach statistical significance (median 10 vs. 5 days; p = 0.2).

Case-patients with LRE infection were more likely than control patients to have spent time in 4 locations in hospital A before the index culture occurred. These locations were 3 specific critical care areas (a medical-surgical ICU, extended postoperative holding area, and ventilator rehabilitation unit; OR 11.0; 95% CI 2.1–61.4) and 1 orthopedic/neurosurgical ward (OR undefined). By multivariate analysis, using forward logistic regression, we identified the following as statistically significant predictors of LRE infection: prior isolation of MRSA (adjusted OR [AOR] 27; 95% CI 4.3–174), duration of hospitalization before index culture (AOR 1.1 per day; 95% CI 1.0–1.2), and duration of preceding linezolid therapy (AOR 1.1 per day; 95% CI 1.0–1.2).

Case–Control Study II

To compare patients with LRVRE and LSVRE infections, we identified 7 case-patients and 28 controls. Case-patients and controls did not differ significantly by age, race, sex, immunocompromising conditions, instrumentation, or proportion of deaths. The length of hospitalization for case-patients compared with that for controls did not reach statistical significance (42 vs. 22 days; p = 0.15). During the previous 12 months, the cumulative days of total antimicrobial drug therapy, and of linezolid therapy specifically, did not differ significantly between case-patients with LRVRE and controls with LSVRE (median 19 vs. 6 days; p = 0.17).

Prevalence Study

Nasal swab samples were obtained from 393 (93%) of the 424 hospitalized patients, and perirectal swab samples were obtained from 388 (92%). MRSA was isolated from 25 (6%) of the 393 nasal swab specimen cultures; linezolid-resistant *S. aureus* was not identified in the cultures. VRE was isolated from 51 (13%) of the 388 perirectal swab specimens, and LRE was isolated from 4 (1%); the 4 LRE isolates also were resistant to vancomycin. Of the 4 patients with LRE, 3 were located on 2 medical-surgical wards and 1 was located in a critical care area.

Laboratory Studies

LRE isolates from 4 case-patients (patients A–D) and 3 environmental isolates from patient B's room (sleep chair, blood-pressure cuff, and hospital bed on/off button) after routine cleaning were available for PFGE (Figure 3). The isolates from cultures of blood and rectal swab specimens

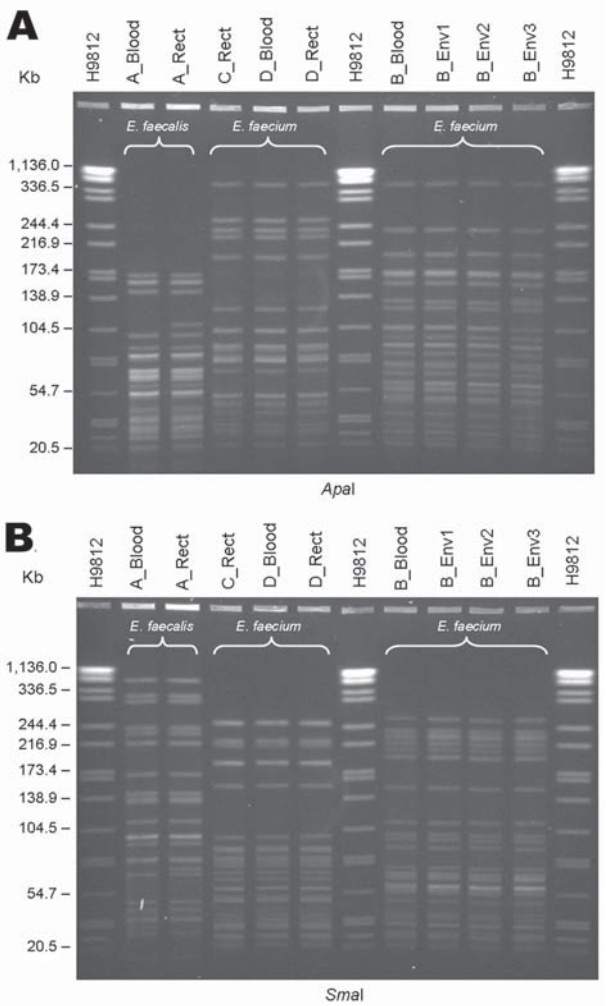

Figure 3. Pulsed-field gel electrophoresis of linezolid-resistant enterococci (LRE) isolates, hospital A, Tennessee. A) Digestion with *Apa*I. B) Digestion with *Sma*I. Isolates labeled A, B, C, and D refer to patients mentioned in the text. Blood, isolate from blood specimen culture; Rect, isolate from perirectal/rectal swab specimen culture; Env, environmental isolate; H9812, *S.* Braenderup H9812 strain (ATCC BAA-664) (*27*) used as size marker.

from patient A (*E. faecalis*) were related but distinguishable (1 and 2 bands different for *Apa*I and *Sma*I, respectively). The environmental isolates (*E. faecium*) were indistinguishable from patient B's blood culture isolate. A third pattern was shared between patients C and D; the isolates of *E. faecium* from blood and rectal swab specimens from patient D were identical.

Antimicrobial Drug Use

Hospital A started using linezolid in April 2000; however, pharmacy purchase data were available only from October 2001 onward. DDD increased from 13/1,000 patient-days in 2001 to 35/1,000 patient-days in 2004 (Figure 4). Most linezolid doses were used outside critical care areas. The drug-usage evaluation identified 177 patients who received linezolid therapy (range of treatment duration 2–30 days). A total of 164 (93%) patients were prescribed linezolid by either infectious disease (118 [67% of 177 total patients receiving linezolid]) or critical care (46 [26% of 177 total patients receiving linezolid]) physicians. Four prescribers (3 infectious disease and 1 critical care) accounted for 144 (81%) patients; 1 prescriber accounted for 64 (36%) patients. Apart from restricting prescription of certain antimicrobial drugs to particular specialty groups, no antimicrobial drug stewardship program was in place.

Infection-Control Policy Review

Patients with known VRE infection were placed on contact precautions. Patients with MRSA infection were placed on contact precautions while in critical care areas. Critical care–area staff performed surveillance cultures of urine and sputum on a weekly basis.

Discussion

We describe risk factors and outcomes associated with a large hospital outbreak of LRE, an emerging pathogen. The licensing-to-resistance time interval for linezolid was brief. Linezolid use increased in response to increases in MRSA and VRE infection in this hospital community and probably because of the drug's convenience compared with vancomycin (e.g., good bioavailability and no requirement for blood-level or renal-dose adjustment). Despite being restricted to use by infectious disease and critical care physicians, the linezolid DDD prescribed by hospital A increased nearly 3-fold in 3 years; most use was outside ICUs. In this investigation, we found that exposure to linezolid any time in the preceding 12 months and the increased cumulative days of linezolid use among case-patients suggest that antimicrobial drug pressure contributed to the emergence of multiple clones of LRE at hospital A. De novo resistance has been documented in several instances in which PFGE comparisons have been made between LSE and LRE isolates in the same patient after exposure to linezolid

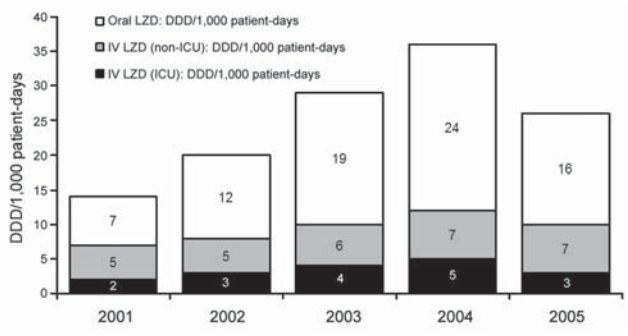

Figure 4. Linezolid (LZD) usage during 2001–2005 at hospital A, Tennessee. Use of oral and intravenous (IV) formulations is shown in defined daily doses (DDD)/1,000 patient days. Data for 2001 and 2005 do not include all 12 months (2001 includes data from October through December; 2005 includes data from January through February). ICU, intensive care unit.

(*10*,*13*,*16*). Our controls consisted of patients with clinical LSE isolates; use of these controls can introduce selection bias, potentially overestimating the OR associated with prior exposure to linezolid (*29*,*30*). One could hypothesize that linezolid protects patients against having subsequent culture results positive for LSE and, thus prevents patients from becoming members of the antimicrobial drug (linezolid)–susceptible control group. The duration of any such potential protection is unknown. However, 4 (50%) LRE case-patients with linezolid exposure had a nonimmediate exposure (>30 days before index culture) to linezolid, compared with 2 (22%) LSE controls.

At hospital A, 8 (53%) of 15 patients with LRE infection had been exposed to linezolid; however, 7 (47%) had not been. This finding has been documented previously with nosocomial transmission of LRVRE in a transplantation unit among 7 patients, of whom 6 were linezolid-naive, and isolated from all had the same pattern on *Sma*I PFGE (*17*). In another study, 2 patients without linezolid exposure acquired LRVRE with identical PFGE patterns (*31*). A clonal outbreak was described by Dobbs et al. (*21*); only 6 (15%) of the patients had received linezolid before contracting LRVRE, and 17 (42.5%) were in a particular ICU before acquiring LRVRE. In our investigation, patient C was linezolid-naive and located in the same critical care unit as patient D (linezolid-exposed); their isolates were identical on PFGE. Isolates recovered from patient B (linezolid-exposed) and from multiple environmental samples from patient B's hospital room after routine cleaning were indistinguishable with 2 enzymes by PFGE, indicating possible patient-to-patient transmission through contaminated fomites or healthcare workers' hands. This pattern was distinguishable from both the patient A pattern and patient C/D pattern. Patient-to-patient transmission is further supported by the clustering

of exposures in time (January–February 2005) and space (3 specific critical care units and an orthopedic/neurosurgical ward).

A strong risk factor for LRE was prior MRSA infection. In 4 (27%) case-patients, linezolid was used to treat prior MRSA infection. However, the relationship between prior MRSA and LRE goes beyond this expected association of prior MRSA and linezolid exposure. Even among those with no linezolid exposure, MRSA was strongly associated with linezolid resistance (OR 23; 95% CI 2.6–272.0). This was confirmed on multivariate analysis (AOR 27). Prior MRSA infection therefore might also be a surrogate marker for patient-to-patient transmission of LRE or increased susceptibility to nosocomial infection.

In this outbreak, patients with LRE infection experienced more illness, were hospitalized longer, and were more likely to die than patients with LSE infection. We cannot determine whether the higher number of deaths were attributable to LRE; additional studies are required, including matching of controls to case-patients on severity-of-illness measures. In addition, our sample size and statistical power were limited by the number of cases of LRE and, in particular, LRVRE.

Infection-control efforts should focus on preventing infections and interrupting patient-to-patient transmission of multidrug-resistant organisms (MDROs) (*32*,*33*). Widespread rise in MDROs (e.g., MRSA) likely contributes to increases in linezolid prescription and LRE. Tracking or restricting linezolid use (e.g., for treatment of invasive VRE or MRSA) might reduce antimicrobial drug pressure and slow down emergence of LRE, which is critical because only a limited number of antimicrobial drugs are available to treat resistant gram-positive infections. Recently published guidelines (*34*) recommend use of additional interventions, such as active surveillance cultures and contact precautions, if either of the following 2 conditions is met: "1) the incidence or prevalence of MDROs are not decreasing despite the use of routine control measures; or 2) the first case or outbreak of an epidemiologically important MDRO (e.g., VRE, MRSA) is identified within a healthcare facility or unit. Facilities should continue to monitor the incidence of target MDRO infection and colonization; if rates do not decrease, implement additional interventions as needed to reduce MDRO transmission." However, some hospitals might not implement active surveillance cultures because of concerns about potential delays in discharging colonized patients to nursing homes. Other obstacles are logistic (e.g., ensuring compliance rates ≥90% for surveillance cultures) and financial (e.g., patients cannot be charged for surveillance cultures, or insufficient infection-control resources might exist). These concerns and obstacles should be addressed; otherwise, the response to 1 emerging resistant infection will breed another emerging infection.

Acknowledgments

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Dr Kainer is an infectious diseases physician and medical epidemiologist at the Tennessee Department of Health, where she is Director of the Hospital Infections and Antimicrobial Resistance Program. Her research interests include healthcare epidemiology, infection control, and antimicrobial drug resistance.

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Address for correspondence: Marion A. Kainer, Tennessee Department of Health, Communicable and Environmental Disease Services, 1st Floor, Cordell Hull Bldg, 425 Fifth Ave N, Nashville, TN 37243, USA; email: marion.kainer@state.tn.us

Person-to-Person Transmission of Nipah Virus in a Bangladeshi Community

Emily S. Gurley,* Joel M. Montgomery,† M. Jahangir Hossain,* Michael Bell,† Abul Kalam Azad,‡ Mohammed Rafiqul Islam,‡ Mohammed Abdur Rahim Molla,‡ Darin S. Carroll,† Thomas G. Ksiazek,† Paul A. Rota,† Luis Lowe,† James A. Comer,† Pierre Rollin,† Markus Czub,§¶ Allen Grolla,§ Heinz Feldmann,§¶ Stephen P. Luby,*† Jennifer L. Woodward,# and Robert F. Breiman*†

An encephalitis outbreak was investigated in Faridpur District, Bangladesh, in April–May 2004 to determine the cause of the outbreak and risk factors for disease. Biologic specimens were tested for Nipah virus. Surfaces were evaluated for Nipah virus contamination by using reverse transcription–PCR (RT-PCR). Thirty-six cases of Nipah virus illness were identified; 75% of case-patients died. Multiple peaks of illness occurred, and 33 case-patients had close contact with another Nipah virus patient before their illness. Results from a case-control study showed that contact with 1 patient carried the highest risk for infection (odds ratio 6.7, 95% confidence interval 2.9–16.8, p<0.001). RT-PCR testing of environmental samples confirmed Nipah virus contamination of hospital surfaces. This investigation provides evidence for person-to-person transmission of Nipah virus. Capacity for person-to-person transmission increases the potential for wider spread of this highly lethal pathogen and highlights the need for infection control strategies for resource-poor settings.

Nipah virus was first identified as the pathogen responsible for outbreaks of encephalitis in Malaysia and Singapore from October 1998 to June 1999 (*1–6*). Fever (97%), headache (61%), and reduced consciousness (55%) were the most common symptoms in Malaysia; case-fatality rate was 40% (*7*). Most case-patients lived on pig farms (95% in Malaysia) (*1*) or worked in abattoirs (100% in Singapore) (*4,8*). Serologic and reverse transcription–PCR (RT-PCR) testing of blood and urine from pteropid fruit bats in Malaysia and Cambodia showed Nipah virus infection, which suggested that these animals were reservoir hosts (*9–11*). During this outbreak, Nipah viruses were also isolated from human respiratory secretions and urine (*2*); however, 2 studies did not find evidence of nosocomial transmission (*12*,*13*).

Subsequent investigations in India and Bangladesh have suggested that Nipah virus may have been transmitted from person to person. During an outbreak in Siliguri, India, in 2001, 45 (75%) of 60 patients, many of them healthcare workers, had a history of hospital exposure to patients infected with Nipah virus (*14*). A case-control study conducted during an outbreak in Meherpur District, Bangladesh, in 2001 showed that persons who lived with or cared for patients during the patient's illness were more likely to become infected with Nipah virus, and patients were more likely to have reported touching secretions of other patients; however, this finding could not be differentiated from common environmental exposures (*15*). During an outbreak in Rajbari District, Bangladesh, in January 2004, case-patients were more likely than controls to have had contact with another patient with Nipah virus illness (*16*). Pteropid bats were also suspected to be the reservoir for the virus in Bangladesh (*9–11*,*15*).

On April 5, 2004, the ICDDR,B and the Institute for Epidemiology and Disease Control Research were alerted to a cluster of 5 persons with fever, headache, confusion, and loss of consciousness in Faridpur District, in western Bangladesh. Nipah virus was the suspected cause of the

*ICDDR,B, Dhaka, Bangladesh; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡Ministry of Health and Family Welfare, Dhaka, Bangladesh; §Public Health Agency of Canada, Winnipeg, Manitoba, Canada; ¶University of Manitoba, Winnipeg, Manitoba, Canada; and #University of Texas School of Public Health, Houston, Texas, USA

outbreak and an investigation began on April 6, 2004. Investigation goals were to identify a reservoir host(s), define the magnitude of the epidemic, and determine the principal modes of transmission. This report addresses the last 2 goals.

Methods

Finding and Defining Cases

We defined suspected case-patients with Nipah virus illness as persons with fever and altered mental status (serious illness) residing or working in the outbreak area or persons who had fever and cough or headache (mild illness) and who were contacts of patients with Nipah virus infection or resided in the outbreak area. Suspected case-patients were identified by visiting area hospitals, conducting door-to-door visits to all homes in the affected area, and tracing contacts of patients with Nipah virus illness. A history of illness and general information about exposures were obtained for each suspected case-patient. Friends and relatives of deceased case-patients served as proxy informants for interviews, and guardians were included in interviews of children <13 years of age. All those who died in the outbreak areas during this time were considered suspected case-patients.

A probable case-patient was defined as a patient with fever and mental status changes who lived or worked in the same village as a confirmed case-patient and from whom either serum or cerebrospinal fluid (CSF) was not available (i.e., because the patient died before specimen could be collected) or from whom a negative result was obtained from a sample collected <10 days after illness onset but collection of subsequent specimens was impossible (*17*). A laboratory-confirmed case of Nipah virus infection was defined by evidence of acute infection shown by immunoglobulin M (IgM) to Nipah virus in serum or CSF. To evaluate the possibility of asymptomatic infections, we asked persons with a history of close contact with a patient with Nipah virus–like illness to provide a blood specimen for serologic testing (*7*,*13*).

Specimen Collection and Laboratory Testing

Acute-phase blood specimens, throat swabs, saliva, and urine were collected from persons with suspected cases. When possible, hospitalized patients underwent lumbar puncture and chest radiography. Convalescent-phase blood specimens were collected from all persons with suspected cases >10 days after illness onset. Acute- and convalescent-phase serum and CSF were tested with an IgM capture enzyme immunoassay (EIA) for IgM and an indirect EIA for IgG by using Nipah virus (Malaysian prototype) antigen S (*18*) at the Centers for Disease Control and Prevention in Atlanta, Georgia, USA. Acute-phase serum, CSF, throat swabs, saliva, and urine were also tested by RT-PCR for viral RNA. RNA was extracted from specimens by using the acid guanidinium–phenol method (*19*). RT-PCR was performed by using a primer set to detect the nucleocapsid gene as described (*20*), the Superscript One Step RT-PCR Kit (Invitrogen, Carlsbad, CA, USA), and standard reaction conditions (*21*). Primers used were NVNF-4: 5′-GGA GTT ATC AAT CTA AGT TAG-3′ and NVNBR4: 5′-CAT AGA GAT GAG TGT AAA AGC-3′. PCR products were subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. Positive PCR results were confirmed by sequence analysis of PCR products.

Case–Control Study

We conducted a case–control study to identify risk factors for transmission of Nipah virus infection. Persons meeting either the probable or confirmed case definition were enrolled as case-patients. All healthy persons including household members and neighbors (residing within 150 m of a case household), were eligible for participation as control participants. Controls were randomly selected from a list of names generated by a census of all households in the affected community and matched to a case-patient (6:1) by sex and age (± 2 years; all case-patients and controls were >4 years of age). They were given a 2-part questionnaire in Bengali. The first part focused on environmental exposures and established whether the participant had had contact with a specific probable or confirmed case-patient(s). The second part focused on types of contact with specific case-patients to elucidate possible modes of transmission from person to person. Proxy interviews were conducted with guardians and companions for case-patients who had died or who were unable to respond and for all case-patients and controls <13 years of age.

We calculated odds ratios (ORs) and 95% confidence intervals (CIs) by using conditional univariate logistic regression that accounted for matched enrollment of case-patients and controls (*22*). To evaluate independent risk factors, we tested all variables with a p value <0.1 from univariate analyses in conditional stepwise forward multivariate logistic regression. Associations were considered statistically significant if p value was <0.05. All statistical analyses were performed with SAS version 9.0 (SAS Institute Inc., Cary, NC, USA).

Environmental Study

In early May 2004, 5 weeks after the outbreak was first recognized, environmental surfaces believed to have a high risk for contamination with Nipah virus were selected for sampling. These included surfaces within hospitals where Nipah virus patients received care, surfaces within homes of confirmed case-patients, trees where bats foraged, date

palm sap collection pots, and fruits that may have been in contact with fruit bats. Surfaces were rolled with sterile, cotton-tipped applicators, which were stored in 500 μL Dulbecco modified Eagle medium supplemented with an antibiotic-antimycotic solution. Viral RNA was extracted from 140 μL of the resulting suspension by using a Viral RNA Minikit (QIAGEN, Mississauga, Ontario, Canada) and eluted in a volume of 50 μL. Nipah virus RNA was detected by using the LightCycler RNA Amplification Kit SYBR Green I (Roche, Laval, Quebec, Canada) with primers for the nucleoprotein gene (NPF: 5′-ATCAATCGTGGTTATCTTGAAC-3′ and NPR: 5′CCTCTTCGTCGACATCTTGATC-3′) and with thermocycling and real-time detection performed on a SmartCycler II reaction block (Cepheid, Sunnyvale, CA, USA). Positive results were later confirmed by direct sequencing of amplified products, and sequences were compared with those obtained from patient samples (*23*).

Participants and Ethical Considerations

Because this was an outbreak investigation, protocols did not undergo formal institutional review. The Bangladesh Ministry of Health and Family Welfare requested this investigation and reviewed and approved all protocols. Participation in these studies was strictly voluntary and informed consent was obtained from all participants; for those <18 years of age, individual and parental consent were obtained.

Results

Defining Cases

We identified and collected specimens from 210 suspected case-patients, of whom 32 had fever and altered mental status. Thirty-six case-patients were identified, including 4 who had a febrile illness without altered mental status. Twenty-three (64%) were laboratory confirmed and 13 (36%) were classified as probable; 27 (75%) died. Serum specimens were available from 27 of 36 case-patients, 4 of which had no detectable antibodies to Nipah virus. These specimens were collected <6 days after illness onset. All 4 patients died before a second specimen could be obtained; therefore, they were classified as probable case-patients. Nine patients who had encephalitis-like illnesses and who resided in the outbreak area died before diagnostic specimens could be collected; they were also classified as probable case-patients. No asymptomatic cases of Nipah virus infection were documented in contacts of Nipah virus patients who consented to provide a blood specimen (n = 20). Results of sequencing RT-PCR products from throat swab, saliva, and urine samples of 9 patients were consistent with serologic data, which indicated that Nipah virus was the etiologic agent of this outbreak (*23*).

Contact Tracing

Probable and confirmed case-patients were identified in 7 villages in Faridpur District; dates of onset of illness ranged from February 19 to April 17, 2004 (Figure 1). Thirty-three (92%) of the 36 case-patients had had close contact with another ill person before they became ill (Figure 2). Five cases (patients A, B, F, G, and EE) appeared to be associated with secondary and tertiary person-to-person transmission of Nipah virus. We present some examples of close contact.

Four of Patient A's caregivers who resided in his village became ill after their contact with him; they were his mother (patient D), his son (patient E), his aunt (patient B), and a neighbor (patient C). They became ill 15–27 days after patient A became ill. Patient B received care from her brother (patient F), who lived in a village ≈30 minutes from her village and who became ill 13 days after patient B.

Patient F became ill after he had returned to his village. As shown in Figure 2, 22 (61%) of 36 cases in this outbreak had contact with patient F before they became ill. Patient F was a local religious leader. Many persons in his family and his followers had close contact with him during his illness. Eight (80%) members of his 10-person household became infected with Nipah virus 6–13 days after his illness onset. Two of patient F's brothers (patients T and U), both of whom lived ≈2 hours away, visited him for 6 hours on the day he died. They developed serious illness 7 (patient T) and 11 days (patient U) days later, and both died. Patients Y (patient F's daughter) and Z (patient F's son-in-law), who lived 1 hour away from patient F, became ill ≈1 week after 1 multiple-hour visit with patient F late during his illness; this was their only reported contact with a Nipah virus patient. In 11 other close contacts of patient F, including family and religious followers, Nipah virus infection developed 6–14 days after his illness onset.

Patient G, a follower of patient F, moved to his family's house in an adjacent village to receive care after becoming ill. Patient H became ill ≈9 days after physically supporting patient G while walking to patient G's family's house. A rickshaw driver, patient I, who helped carry

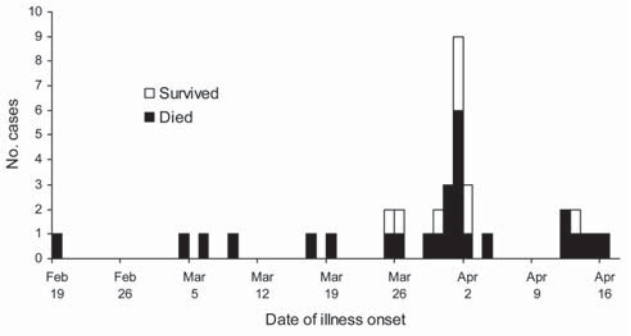

Figure 1. Dates of illness onset during a Nipah virus outbreak in Faridpur District, Bangladesh, 2004.

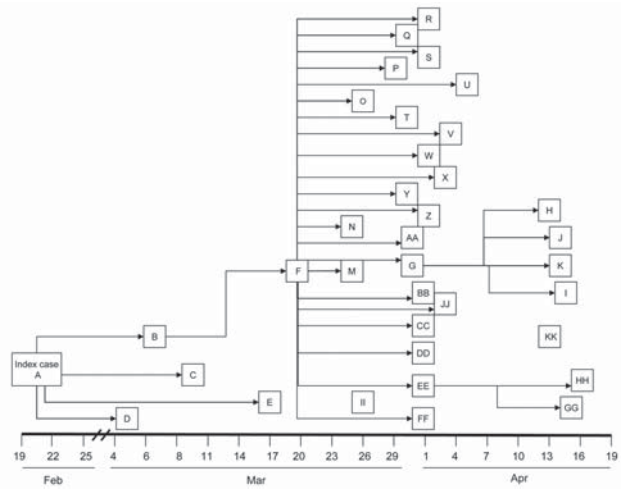

Figure 2. Chain of person-to-person transmission with dates of onset of illness during a Nipah virus outbreak, Faridpur District, Bangladesh, 2004. Letters identify individual patients. Patients KK and II had no known contact with any ill patient before their illness.

and transport patient G to the hospital as his condition deteriorated, became sick 10 days after this exposure and later died. Patient G's father (patient J) and sister-in law (patient K) cared for him during his illness; both became severely ill 2 weeks after patient G's illness onset; only patient K survived.

Patient GG visited his wife and daughter, both hospitalized as suspected Nipah virus patients, at the local healthcare facility. Patient GG spent the night in the hospital and shared a bed with patient EE (a common practice in Bangladesh), who was a male friend, and a suspected Nipah virus case-patient. Although tests on patient GG's wife and daughter did not detect Nipah virus infection, patient EE's infection was laboratory confirmed. Patient GG became ill 10 days after this contact and died.

Patients II and KK had no known contact with any ill patient before their illness and were distinct from other case-patients in that they were not friends, relatives, or followers of patient F and lived outside the affected villages. No cases of Nipah virus illness among healthcare workers were reported to authorities during this outbreak.

Case–Control Study

Thirty-four of the 36 case-patients were enrolled in the study and matched to 6 controls each (n = 204) by age and sex (Table). Two case-patients (patients JJ and KK; Figure 2) were not included in the study because of logistic constraints. Patient JJ was away from her home when the questionnaire was administered. Patient KK was a policeman who had 2 residences and traveled frequently while on duty; we were therefore unable to identify appropriate proxies for his interview.

Ten variables were significantly associated with Nipah virus infection in univariate analysis (online Appendix Table 1, available from www.cdc.gov/EID/content/13/7/1031-appT1.htm). Having had close contact (touching or receiving a cough or sneeze in the face) with patient F placed a person at greatest risk of acquiring Nipah virus infection (OR 6.7, 95% CI 2.9–16.8, p<0.001). Having had any contact with someone who later died, had a fever, was unconscious, or had respiratory difficulties was also associated with illness. Having avoided any contact with someone who later died was negatively associated with illness. Having had a household member harvest date palm sap was the only environmental exposure associated with an increased risk for infection. Having visited the home village of the index case-patient was also associated with illness. However, the only exposure variable that remained significant in multivariate analysis was having had contact with patient F (OR 5.6, 95% CI 1.79–17.24, p = 0.003).

Univariate analysis of risk factors specifically associated with types of contact with patient F was conducted. Fourteen of 42 exposure variables were associated with illness (online Appendix Table 2, available from www.cdc.gov/EID/content/13/7/1031-appT2.htm). Having had close body contact and having spent longer periods of time with patient F were associated with illness. Having kept a certain distance from patient F and having washed hands after contact with him were negatively associated with illness (online Appendix Table 2). Despite multivariate analysis of risk factors associated with type of contact with patient F, insufficient sample size (n = 50) resulted in overfitting of the model and spurious results.

Environmental Study

A total of 468 environmental specimens were collected by swabbing; 137 from walls, bed frames, mattresses, and floors of 2 Faridpur hospitals; 57 from surfaces and utensils of case-patient residences; 150 from trees where bats forage and from fruits; 98 from bat excreta; and 26 from other sites. Eleven positive specimens were collected from

Table. Characteristics of 34 case-patients infected with Nipah virus in case–control study, Bangladesh, April–May 2004

Characteristic	No. (%) case-patients
Sex	
Male	20 (58.8)
Female	14 (41.2)
Age group, y	
1–15	4 (11.8)
16–24	1 (2.9)
25–40	19 (55.9)
41–60	9 (26.5)
>60	1 (2.9)
Adults vs. children	
≤15 y of age (children)	4 (11.8)
>15 y of age (adults)	30 (88.2)

the surrounding wall and bed frame where a confirmed case-patient (patient Z) had been hospitalized on April 6, ≈5 weeks before environmental samples were collected. No other patients with encephalitis were known to have used that bed after patient Z's hospitalization. The wall and bed frame were visibly soiled, and hospital staff reported that they had not been cleaned since the outbreak. Samples from these areas showed evidence of Nipah virus RNA. Sequences of PCR products were identical to sequences of Nipah viruses isolated from patient HH during the outbreak (*18*). No samples from case-patient residences, bat-feeding sites, or fruits were positive.

Discussion

This investigation provides compelling evidence for person-to-person transmission of Nipah virus. Exposure histories of infected patients and the epidemiologic curve, which demonstrates multiple peaks of illness onset during this outbreak, suggest that Nipah virus was transmitted by person-to-person contact. Contact tracing documented Nipah virus illness after brief, yet close contact, with other persons infected with Nipah virus. Findings from the case-control study, which showed a 6-fold increased risk for infection for those who reported contact with patient F, a negative association with illness after handwashing, and specific exposures to ill persons linked to transmission, confirm that exposure to ill persons spread the outbreak.

Person-to-person transmission of Nipah virus is biologically plausible. Other paramyxoviruses that infect humans, including human parainfluenza viruses 1–4, measles virus, and mumps virus, are also transmitted from person to person. Nipah virus has been isolated from human respiratory secretions, including those of cases from this outbreak (*2*,*23*). Furthermore, we identified that direct exposure to respiratory secretions of patients with Nipah virus illness was associated with infection during this outbreak.

The number of villages affected by the outbreak increased as persons traveled in and out of the affected areas to visit family members. This movement led to new infections in caregivers from other villages and increased the number of villages affected. Similar to transmission of severe acute respiratory syndrome (*24*), transmission of Nipah virus infection was not associated with all case-patients; however, 1 case-patient, patient F, was associated with 22 subsequent Nipah virus infections. Although host biologic factors may have resulted in increased viral shedding, leading to higher attack rates, the social status of patient F in the community enabled closer contact with more persons during his illness and more opportunity to transmit infection.

During the outbreak in Siliguri, India, 33 healthcare workers and hospital visitors became ill after exposure to hospitalized patients with Nipah virus illness, suggesting nosocomial infection (*14*). In Malaysia and Singapore, contact with pigs was associated with infection; healthcare worker studies showed that the risk for nosocomial transmission was low (*6*,*8*,*12*,*13*,*25*). Absence of person-to-person transmission in Malaysia and Singapore could be due to differences in patient care practices, host susceptibility factors, or strain variation (*23*).

Detection of Nipah virus RNA on hospital surfaces demonstrates that infected patients shed virus into the environment, which could provide an opportunity to transmit Nipah virus to others. However, how long the virus will remain infectious in the environment is not known, and no evidence from this investigation indicates that type of transmission occurred. Despite the absence of healthcare worker infection during this outbreak, enhanced infection control practices, such as patient isolation and use of gloves and masks, likely had little protective effect because they were not implemented until late in the outbreak. This outbreak provides evidence that 1 person (patient GG) was infected during a hospital visit while sharing a bed with a confirmed case-patient. Nosocomial transmission of Nipah virus was reported during the outbreak in Siliguri, India (*14*). Efforts are needed to develop and disseminate reasonable guidelines for infection control and prevention for healthcare facilities and communities in resource-poor settings, especially when one considers our finding that handwashing prevents disease transmission.

Transmission of Nipah viruses to humans during this outbreak appears to have been bimodal. Fruit bats continue to be the only identified primary reservoir for the virus (*9–11*,*15*). In contrast with the Malaysia and Singapore experience, no intermediate hosts have been identified in Bangladesh (*15*). During this outbreak, the introduction of virus into human(s) from an unknown initial source appears to have been followed by person-to-person transmission. Three case-patients had no known contact with a sick patient before onset of illness; these case-patients may have been infected through exposure to virus-contaminated bat saliva, urine, or feces or through contact with some unknown intermediate host. These cases provide further evidence that sporadic cases of Nipah virus infection continue to occur in Bangladesh (*26*).

Selection of probable case-patients in this study could have been biased toward finding person-to-person transmission because probable case-patients by definition lived in the same area as confirmed case-patients, which increased the likelihood that they had had contact with one another. To rule out the possibility that this had an effect on our case-control study findings, we analyzed our data by using only confirmed cases; there were no major differences in findings compared with analysis that used confirmed and probable cases. Our findings are also limited by recall bias. Family and friends were often asked to provide information

about a deceased patient weeks after their illness (data for 5 patients were collected >1 month after illness onset). When possible, medical records were used to supplement patient reports of illness history, but often these records were incomplete or nonexistent. However, the investigation began just days after onset of illness in most patients, which provided for optimal recall of events. In addition, case-patients or their proxies might have more carefully considered their exposures than controls. However, all community members were aware of, and concerned by, the outbreak, and we believe it is unlikely that any control did not remember their exposure to case-patients in their community.

Capacity for person-to-person transmission increases the risk for wider spread of this highly lethal pathogen. In an impoverished, densely populated country such as Bangladesh, a lethal virus could rapidly spread before effective interventions are implemented. This spread would provide the seed for a substantial regional or global public health problem and highlights the need for local surveillance, outbreak detection and response, and rapid laboratory diagnostics. Sustained, long-term research is needed to characterize the reservoir of the virus and mechanisms for animal-to-animal, animal-to-human, and human-to-human transmission; clarify climatologic and other environmental factors linked to transmission; and define viral epitopes potentially linked to virulence and transmission. Effective infection control practices appropriate for resource-constrained healthcare systems and communities are urgently needed.

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Ms Gurley works with the Program on Infectious Diseases and Vaccine Sciences at the ICDDR,B, and has participated in 3 Nipah virus outbreak investigations in Bangladesh. Her research interests include infectious disease and disease prevention and control in resource-poor settings.

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Address for correspondence: Emily S. Gurley, Program on Infectious Diseases and Vaccine Sciences, ICDDR,B, GPO 128, Mohakhali, Dhaka 1212, Bangladesh; email: egurley@icddrb.org

Effects of Internal Border Control on Spread of Pandemic Influenza

James G. Wood,*† Nasim Zamani,† C. Raina MacIntyre,*† and Niels G. Becker‡

We investigated the capacity of internal border control to limit influenza spread in an emergent pandemic in the context of Australia, a country with a low-population density and geopolitical boundaries that may facilitate restrictions. Mathematical models were used to study the time delay between epidemics in 2 population centers when travel restrictions were imposed. The models demonstrated that population size, travel rates, and places where travelers reside can strongly influence delay. The model simulations suggested that moderate delays in geographic spread may be possible with stringent restrictions and a low reproduction number, but results will be sensitive to the reproduction number and timing of restrictions. Model limitations include the absence of further importations and additional control measures. Internal border control may have a role in protecting domestic centers early in a pandemic, when importations are sparse. Our results may be useful for policymakers.

Commercial air travel has increased dramatically since the last pandemic of influenza (*1*). The number of international tourist arrivals recorded worldwide in 2004 was 763.2 million; 43% of these arrivals were by air (*2*). This increase in international travel has heightened the risk for the global spread of infectious diseases (*1*).

Long-distance domestic routes also carry high volumes of travelers: an estimated 40.4 million passengers traveled on Australian domestic airlines in the year ending June 30, 2005 (*3*), and 660 million traveled on US domestic airlines during 2005 (*4*). Rapid and accessible long-distance transportation facilitates the geographic spread of diseases, even those, such as influenza, that have a short incubation period (*5*).

*National Centre for Immunisation Research and Surveillance of Vaccine Preventable Diseases, Sydney, New South Wales, Australia; †The University of Sydney, Sydney, New South Wales, Australia; and ‡Australian National University, Canberra, Australian Capital Territory, Australia

If an influenza pandemic emerges, the first attempts to control its spread are likely to be made at its source, as suggested in recent modeling papers (*6*,*7*). However, if these strategies fail, individual governments will need to implement strategies to manage the pandemic when it arrives on their borders. In addition to well-publicized options for control, including antiviral prophylaxis and quarantine (*6*,*7*), travel restrictions, both external and internal, may play a role in reducing the geographic spread of the virus (*8*–*11*)

Restrictions on travel can have a sizeable economic and social impact, as seen in affected nations during the crisis with severe acute respiratory syndrome (SARS). In many countries, stringent travel restrictions will not be feasible because of high population densities and highly connected networks of transportation, infrastructure, and trade. These caveats do not apply to Australia, an island comparable in size to the United States but with a population of only 20 million. This population is concentrated in 5 large cities, along with smaller centers, primarily along its eastern and southern coastlines. These centers are widely separated; travel between them is primarily by air. During the 1918 pandemic, Australia delayed the onset of the pandemic by 1 year by imposing external border control (*12*).

We used mathematical models to make predictions about the effectiveness of travel restrictions and to explore the sensitivity of these predictions to disease and demographic factors. Typically, modeling studies of influenza spread are focused on predicting international or national spread between major hubs on the global air-transportation network (*8*,*9*,*13*), which is certainly important. In contrast, we examine the effects of travel restrictions on 2-city routes with differing characteristics. This simpler setting allows a more detailed exploration of how the delay between epidemics in 2 connected locations depends on travel restrictions, population sizes, travel rates, residence

of travelers, and the transmissibility of the influenza virus, with relevance to large and small centers. The analysis assumes case-patients arriving from overseas have a negligible effect, so the results apply primarily during the early stage of a pandemic. Simulating the effect of internal travel restrictions in Australia is relevant to countries with similar demographic characteristics, such as Russia, Canada, and New Zealand. The aims of our analysis were to explore the role of travel restrictions in slowing the geographic spread of an influenza pandemic and to simulate the effects of such restrictions in the context of Australia.

Methods

Two simple scenarios (Figure 1A) were used to assess the likely impact of travel restrictions on the spread of a pandemic in Australia. In the first, it was assumed that the initial cases occurred in Sydney. The growth of this epidemic and its resultant spread to Melbourne in the presence of travel restrictions were simulated. This scenario is indicative of the spread to other large centers with similar travel volumes. In the second scenario, the initial case was assumed to occur in Darwin, a smaller Australian city in close proximity to Southeast Asia, and the growth of this epidemic and spread to Sydney were simulated. The Darwin-to-Sydney scenario, with a comparatively low travel volume, represents the situation of containing the epidemic within a smaller town through the use of travel restrictions. Key parameters and assumptions are summarized in Table 1.

Data

Average daily volumes of domestic air travel between Sydney, Melbourne, and Darwin were obtained from the Australian Domestic Airline Activity report (*17*). Only direct flights were considered. Seasonal variations in the volume of air traffic were not taken into account. Approximately 78% of the traffic from Sydney to Melbourne and 70% of the traffic from the Northern Territory to the eastern Australian states is by air (*18*).

As a separate indicator of travel volumes that incorporates the average length of stay and information on the origin of travelers, we used survey estimates of nights stayed by domestic visitors to the 3 study destinations (Melbourne, Sydney, and Darwin). The data were obtained from the state government tourism websites for Victoria (*20*), New South Wales (NSW) (*19*), and the Northern Territory (*21*). Because details on visitor origin were only obtained at the state level, we assumed that each person in that state would make an equal contribution to visitor nights in the destination city. These values were then used to estimate the proportions of the travel volume due to each of the 2 cities on a route and to modify force of infection calculations by incorporating the average length of stay. The travel rates (weighted by length of stay) used in the simulations are provided in Table 1. Demographic data on cities and states were acquired from the Australian Bureau of Statistics population estimates for 2004 (*16*).

Model Structure

Simulations of influenza epidemics were computed by using a stochastic SIR model, in which the population is separated into 3 mutually exclusive classes: susceptible (*S*), infectious (*I*), and recovered (*R*). A stochastic model can capture random variation near the beginning of an epidemic, when the number of infectious persons is small. Homogeneous mixing is assumed, i.e., all susceptible members of the population in a city are equally likely to be infected by a given infectious person.

A schematic of the model is given in Figure 1B, and the defining equations are presented in the online Appendix, part A (available from www.cdc.gov/EID/content/13/7/1038-app.htm). The model evolves in discrete time, with the step length equal to 1 day. This time frame accords with real-life epidemics, for which incidence and other epide-

Figure 1. Schematic of travel locations and model. A) Model schematic showing the SIR (susceptible, infectious, and recovered) classes and travel connecting the cities; B) locations of the cities and routes used in the model; C) the form of the 2 infectivity functions used to simulate the infectivity of persons over the course of their infection.

Table 1. Summary of parameter values, assumptions, and sources used in models of the effect of travel restrictions on pandemic influenza in Australia*

Variable/concept	Value (range)/assumption	Source/interpretation
Reproduction no. (R_0)	1.5–3.5	Mills (*14*)
Infectivity function (ρ)	Flat or peaked†	Longini, Ferguson (*7*,*8*)
Latent period	1 (1–2 in sensitivity analysis) d(s)	Ferguson (*6*)
Infectious period	5 d	Literature suggests 4–7 d in adults (*6*,*7*)
Mixing	Homogenous (within city)	Modeling literature (*15*)
Propensity to travel	Everyone equal	Assumption
Populations	Sydney (4.2 million), Melbourne (3.6 million), Darwin (110,000)	ABS figures (*16*)
Travel rate‡ Sydney ↔ Melbourne (weighted by stay length)	(4.7×10^3, 8.9×10^3)	BTRE figures (*17*,*18*), NSW, and Victoria Tourism reports (*19*,*20*)
Travel rate‡ Sydney ↔ Darwin (weighted by stay length)	(9.2×10^4, 4.4×10^3)	BTRE figures (*17*,*18*), NSW, and NT Tourism reports (*19*,*21*)
Travel restrictions	20%,10%, or 1% of current levels	Assumption
Time between 20 current cases in city 1 and city 2 (T_{20})	Random variable (T_{20}), different for each simulation. Median value over all simulations is given by m_{20}.	Output variables used to measure effect of travel restrictions

*ABS, Australian Bureau of Statistics; BTRE, Bureau of Transport and Regional Economics; NSW, New South Wales; NT, Northern Territory.
†See Figure 1, panel C, for shapes used.
‡This assumes a constant travel rate over the year with no seasonal variation in travel volumes.

miologic data are usually recorded daily. The discrete time structure simplifies the introduction of a variable infectivity profile, incorporating a latent, noninfectious period and a changing degree of infectivity for each person during the course of his or her illness. This feature of the model is supported by virus-shedding studies (*22*) and enables us to contrast the effect of a highly peaked infectivity profile, similar to that used by Ferguson et al. (*6*), with the effect of a constant infectivity profile (*7*), as depicted in Figure 1C.

A key factor governing the effectiveness of our travel restrictions is the average doubling time of the attack rate during the early stages of the epidemic, when growth is exponential. The doubling time is determined by the basic reproduction number (R_0), defined as the average number of secondary infections due to a single primary infected person in a completely susceptible population, and the form of the infectivity profile. The infectivity profile primarily influences the growth rate through the mean time (or serial interval) between cases: ≈2.8 days for the peaked infectivity function and 4 days for the flat infectivity function used here. The doubling time depends linearly on the serial interval so that epidemics that use the peaked infectivity profile double in size almost 1.5× as quickly as epidemics that use the flat infectivity profile, for the same value of R_0. The infectivity profile and R_0 depend on properties of the pathogen and on social, environmental, and genetic factors.

Although influenza appears to be a highly infectious disease, with regular winter epidemics, this is largely due to its short incubation period and genetic drift, which nullifies preexisting immunity. Thus, literature estimates of the effective reproduction number for influenza are typically <4 (*14*) (whereas for measles R_0 is 20 [*15*]), although in localized outbreaks it can be considerably higher (*23*). We take R_0 to be in the range 1.5–3.5, which corresponds to attack rates of 58%–97% (including subclinical infections) in a population without prior immunity or behavioral changes in response to the pandemic.

The total period of infection, including latent period, was assumed to be 6 days (*7*). For each infectivity profile, the latent period was ≈1 day, which is at the low end of literature estimates (other researchers have used values of 1–4 days [*8*,*9*,*24*]). Spread from city to city is incorporated by assuming that each person is equally likely to travel; the daily travel rates were estimated from the data sources described above. This assumption was pessimistic, since symptomatic infected persons may not travel, but it did not greatly influence the results (online Appendix, part A).

Travel restrictions were implemented as a reduction of the rate of all forms of travel. For this analysis, reductions of 80%, 90%, and 99% were compared with the base case of unrestricted travel. The values of 80% and 90% might be realistic reduction targets, whereas the value of 99% indicates what near-perfect compliance might achieve. Travel restrictions were switched on in the model at some time (measured in weeks) after the initial case occurred and remained on for the rest of the simulations.

The principal measure used in this analysis for gauging the effect of travel restrictions is T_{20}, the delay between the epidemic's becoming established in city 1 and taking off in city 2. We considered the outbreak to have taken off in a city once there were 20 current infectious cases—hence, the notation T_{20} for the delay between the epidemics. This choice conveniently limited comparisons to simulated epidemics that do take off. Since the model is stochastic, T_{20} is random, and the results shown in the graphs are for m_{20}, the median value for outbreaks that take off. Ranges, when given, cover 90% of outbreak simulations.

The simulations were run with MATLAB version 7.04 (The MathWorks, Natick, MA, USA) with Poisson random variables simulated by the *poissrnd* function in version 5.02 of the Statistics Toolbox (MathWorks). Our results are based on 10,000 runs of the model.

Motivated by the results of the simulation study, we then analyzed the effects of city size and travel rates by using a deterministic approximation of the above model (details given in the online Appendix, part B). This approximation has the advantage of being much simpler to use in analyzing sensitivity to these factors, while reproducing the average behavior of the stochastic model.

Results

Scenario 1 (Sydney to Melbourne)

The median and mean numbers of days until there are 20 infectious persons in Sydney for an epidemic that began with 1 infectious person in Sydney on day 0 are presented in Table 2. Figure 2 illustrates how m_{20}, the median time between the day when the number of infected persons first reached 20 in Sydney and the day when the number of infected persons first reached 20 in Melbourne, depends on R_0, the form of the infectivity profile, and the timing and severity of travel restrictions.

Each of the graphs covers 1 of the 6 combinations of the 3 values of R_0 and 2 infectivity profiles. The 4 curves shown on each graph describe the median values for each of the 4 levels of travel restrictions (none, 80%, 90%, and 99%), applied at delays from importation of the first case from 0 to 6 weeks (8 weeks for $R_0 = 1.5$). The gray panes highlight the time during which the epidemic grows from 20 to 1,000 cases in Sydney.

The travel restrictions are most effective for the optimistic assumption $R_0 = 1.5$ and constant infectivity (Figure 2A). Figure 2B and C more closely resemble the epidemic growth rates used in recent modeling papers (*6*,*7*). In Figure 2B ($R_0 = 1.5$, peaked infectivity), an increase in m_{20} from 22 to 32 days is seen for 80% restrictions, with a further increase to 52 days for 99% restrictions, if applied immediately. These improvements appear robust for delays of up to 4 weeks, but in fact a sizeable proportion of the simulations have spread to Melbourne by this point. This effect is illustrated in Figure 3A and B, in which we compare the full distribution of T_{20} in the presence of 99% travel restrictions applied at the 2- and 4-week marks, respectively. Both distributions are bimodal, but in Figure 3B, the first mode is substantial. This difference arises because a large proportion of simulated outbreaks spread to Melbourne between the 2- and 4-week marks for this combination of disease parameters, a finding that emphasizes that timing can be critical for the success of travel restrictions. Under the pessimistic assumption of $R_0 = 3.5$ and peaked infectivity, the impact of travel restrictions is muted, and a delay of just 2 weeks renders the restrictions ineffective.

Scenario 2 (Darwin to Sydney)

For an epidemic originating in Darwin, the median times until there are 20 infectious persons in Darwin are almost identical to those for scenario 1 (Table 2), although the 90% ranges are a little wider. In this scenario, m_{20} is the median time between the first day on which there are 20 infected persons in Darwin and the first day on which there are 20 currently infected persons in Sydney. The effects of R_0, the infectivity function, and the delay in and severity of travel restrictions are captured in Figure 4.

These results, presented in the same format as Figure 2, show 2 key differences from those in scenario 1. The median delay, m_{20}, is shorter in scenario 2, given the same combination of disease parameters, as is the time interval over which restrictions can be applied effectively. This finding appears counterintuitive because the volume of travelers on the Darwin-to-Sydney route is much smaller than that on the Sydney-to-Melbourne route.

By using the simpler model described in the online Appendix, part B, we performed a sensitivity analysis (online Appendix Figure, available from www.cdc.gov/EID/content/13/7/1038-appG.htm) on the effect of city size and travel rates on epidemic spread. This analysis implies that in scenario 2, in which there is a large difference in population size (Darwin:Sydney ≈1:40), infection of susceptible travelers from Sydney is the primary reason for the rapid intercity spread, despite the lower rate of travel for Sydney residents on this route. The online Appendix Figure, panel B, shows that this effect would be reduced if the rate at which Sydney residents travel to Darwin were much lower than that for Darwin residents traveling to Sydney. Such a reduction could be achieved by applying tighter restrictions on Sydney-based travelers.

Table 2. No. days for an influenza epidemic beginning in Sydney to total 20 currently infectious cases*

	Constant infectivity profile†			Peaked infectivity profile†		
R_0	Median, d	90% range, d	Mean, d	Median, d	90% range, d	Mean, d
1.5	24	13–46	25.9	15	8–31	16.4
2.5	12	8–21	13.0	8	5–14	8.4
3.5	9	6–14	9.5	6	4–10	6.2

*R_0, reproduction number.
†The constant infectivity profile assumes that a person is equally infectious throughout their infectious period; the peaked infectivity profile assumes that they are most infectious early in the infectious period (see Figure 1, panel C, for the profiles used).

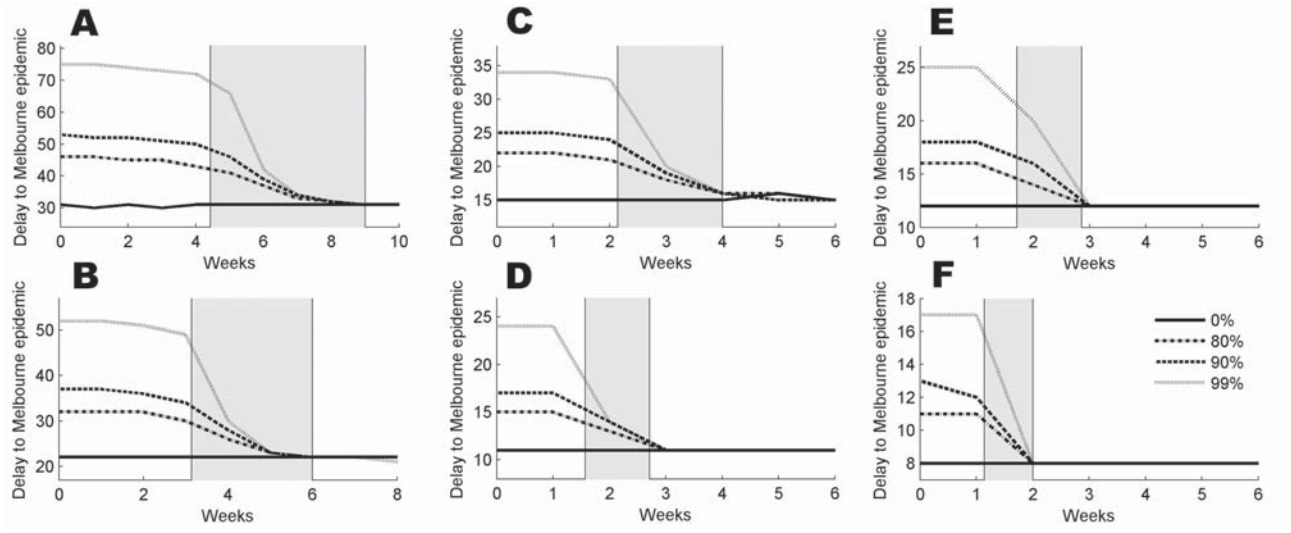

Figure 2. For an epidemic beginning in Sydney, the value of the median time delay, m_{20}, in the presence of travel restrictions applied at a delay of 0–6 weeks (10 and 8 weeks in [A] and [B], respectively). Assumptions are A) reproduction number (R_0) = 1.5, constant infectivity profile; B) R_0 = 1.5, peaked infectivity profile; C) R_0 = 2.5, constant infectivity profile; D) R_0 = 2.5, peaked infectivity profile; E) R_0 = 3.5, constant infectivity profile; F) R_0 = 3.5, peaked infectivity profile. The gray panes cover the periods when the epidemic grows from 20 to 1,000 infected people in Sydney; dotted, dashed, dash-dotted, and solid lines correspond to 99%, 90%, 80% and no travel restrictions, respectively.

The ratio of city populations also influences the time interval when restrictions can be applied effectively. Travel restrictions were less effective if applied after the time at which there were 20 current cases in Darwin (Figure 4). This feature was illustrated by Figure 3C and D which show the full distributions of T_{20} for 2- and 4-week delays in restrictions, respectively. For a 2-week delay (Figure 4C), most outbreaks were delayed but a sizeable minority were not. A 4-week delay (Figure 4D) nullified any impact of the restrictions for this scenario. If, however, travel restrictions were applied immediately after the first case was detected, the increase in T_{20} due to restrictions was almost identical to the increases described in scenario 1.

Now consider a situation in which a small isolated center (town A, population 1,000) attempts to remain pandemic free. Let us assume that on any given day, N visitors stay in the town, and N town members visit pandemic-affected regions. A simple stochastic model of disease spread (online Appendix, part D) can predict the probability that the outbreak can be kept out of town A in terms of N and R_0 (online Appendix, part D). Predictions from this model agree well with simulations, as shown in the online Appendix Figure, panel C. These results indicate that travel restrictions are likely to prevent an outbreak if N is reduced to ≈1/10 per day.

Sensitivity to Other Factors

The sensitivity of the results to the duration of infection and form of the infectivity function were entirely a result of the change in the epidemic growth rate. If, for example, an additional day of latent infection were added, then the delays in spread, when the flat and peaked infectivity functions were used, were ≈25% and ≈37% longer, respectively, which is a considerable effect. However, epidemic

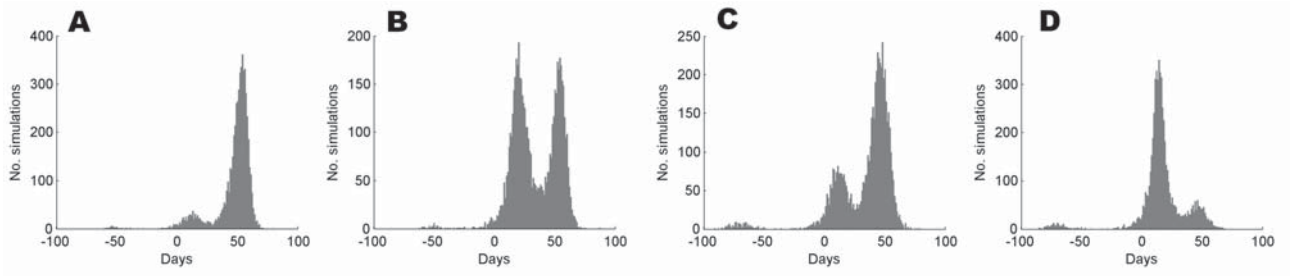

Figure 3. Distributions, based on 10,000 simulations, of the time delay, T_{20}, given reproduction number (R_0) = 1.5 and the peaked infectivity function, with 99% travel restrictions imposed in scenario 1 (A) and (B) and scenario 2 (C) and (D). Scenario 1 simulates an epidemic beginning in Sydney and spreading to Melbourne. In scenario 2, the epidemic begins in Darwin and spreads to Sydney. In (A) and (C), the restrictions are imposed after 2 weeks; in (B) and (D), they are imposed after 4 weeks.

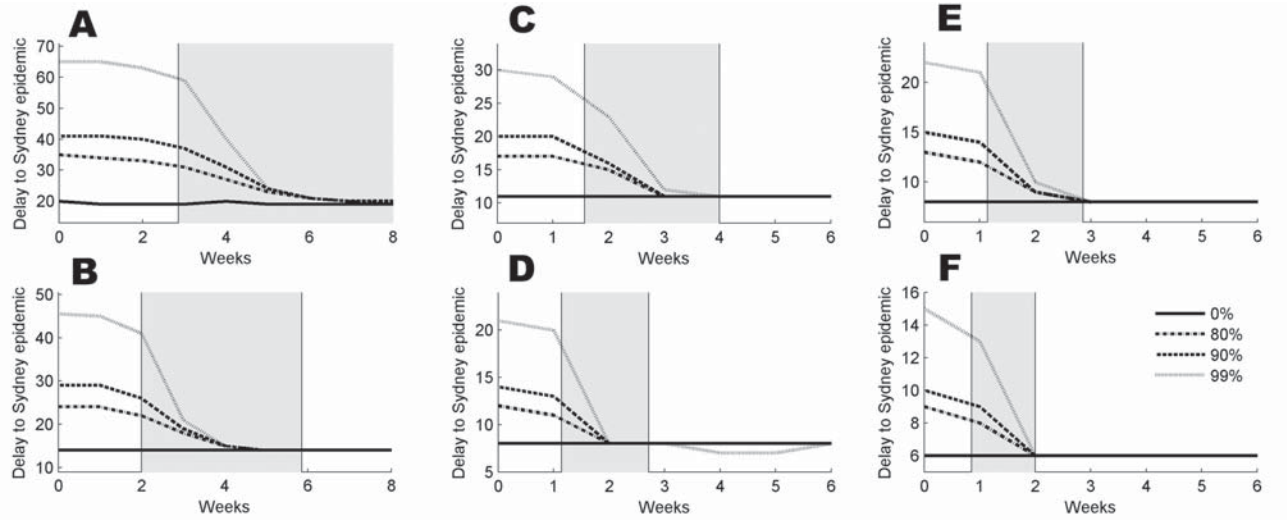

Figure 4. For an epidemic beginning in Darwin, the value of the median time delay, m_{20}, in the presence of travel restrictions applied at a delay of 0–6 weeks (8 weeks in [A] and [B], respectively). Assumptions are (A) reproduction number (R_0) = 1.5, constant infectivity profile; (B) R_0 = 1.5, peaked infectivity profile; (C) R_0 = 2.5, constant infectivity profile; (D) R_0 = 2.5, peaked infectivity profile; (E) R_0 = 3.5, constant infectivity profile; (F) R_0 = 3.5, peaked infectivity profile. The gray panes cover the periods when the epidemic grows from 20 to 1,000 infected people in Darwin. Dotted, dashed, dash-dotted, and solid lines correspond to 99%, 90%, 80%, and no travel restrictions, respectively.

growth rates in past pandemics are typically not consistent with longer latent periods and low values of R_0, so these additional delays should be viewed with caution.

The sensitivity to the estimated travel volumes was relatively weak: increasing or reducing travel by a factor of 2 in each direction increases or reduces the delay by 1.5–7 days, and 4.5 or 2.5 days as compared to data in Figures 2 and 4, respectively. These results are consistent for both scenarios.

Discussion

The simulations we describe showed that although travel restrictions might delay the spread of an influenza epidemic between 2 cities by several weeks, this delay is highly sensitive to assumptions about the transmissibility of the influenza virus. A more surprising result is that the delay is also sensitive to the ratio of city sizes, differences in travel rates, and the originating city. In particular, the modeling suggests that if the epidemic begins in a smaller town, restricting visitors from entering or leaving that town is important.

Moderate delays in the pandemic could be achievable when the epidemic growth rate is low. The growth rate can be estimated from case counts during an epidemic and used in a simple formula to predict the delay due to travel restrictions (online Appendix, part D). These predictions could provide practical estimates of the benefits of longer term travel restrictions based on the first clusters of cases during an outbreak. For smaller communities with low travel rates, the probability of preventing an outbreak can also be estimated (online Appendix, part D), with good agreement with the results of our simulations (online Appendix Figure, panel C). If the estimated growth rate is high (e.g., assumptions used in Figures 2 and 4 with R_0 = 3.5, peaked infectivity), the additional median delay between 20 cases occurring in city 1 and 20 cases occurring in city 2 might be just 3 days, providing little benefit from longer term implementation of travel restrictions

Our results do not account for additional importations. Thus, they are most applicable to the arrival of a pandemic in Australia, while the pandemic outside Australia remains contained or border control is effective. Our simulated delays will be overestimates if additional importations are substantial. Another concern is that stringent travel restrictions may be required for several weeks to maximize delays in spread. Inevitably, such restrictions would cause economic and social disruption, which must be balanced against any benefits from delaying the domestic spread of an epidemic.

If combined with restrictions on overseas travel, restrictions on internal travel may have a role in pandemic control, even for major centers. However, the economic impact of restrictions in major centers could be enormous, with severe consequences for service and travel industries, as seen in the SARS crisis (*25*), and the potential to affect trade and other sections of the economy. Some of the benefits and costs of reduced travel may also accrue without restrictions, with persons avoiding travel because of perceived risks. Our modeling suggests that travel restrictions could have a greater effect in more isolated communities that lack international ports.

The travel restrictions we discussed have been examined in isolation, without consideration of other disease control measures. Other measures could lower the effective value of the reproduction number, or even curtail the epidemic; in these circumstances, reducing all travel by only 80% might be beneficial. Alternatively, if the R_0, is much higher than used here (*23*), internal travel restrictions would be ineffective. Limitations of our modeling approach are summarized in Table 3.

The key points in our study are that delays induced by internal border control are strongly influenced by epidemic growth rates and demographic factors such as the relative sizes of cities, travel rates, and the origin of travelers. When used without other control measures, stopping at least 99% of travel would be required to significantly increase time available for vaccine production and distribution. Although any delay in spread might be attractive for logistical purposes, the economic impact of such restrictions may be prohibitive if sustained for more than a few days. In view of these points, the situation in which they might be most applicable for extended use is in the protection of small, relatively isolated centers.

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Dr Wood is a mathematician in the field of infectious disease modeling at the National Centre for Immunisation Research and Surveillance in Sydney, Australia. His current research interests are in influenza and other vaccine-preventable diseases, as well as underlying mathematical techniques used in modeling.

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Table 3. Limitations and effects of modeling effects of border control on pandemic influenza, Australia

Limitations	Effects
Reproduction number (R_0) and infectivity function for pandemic influenza are unknown.	Larger R_0 and a shorter average time between infections would reduce effectiveness of restrictions.
Further importations not considered.	Frequent importations would greatly reduce benefits of internal restrictions for cities with international airports or ports.
Other control measures (pharmaceutical and social distancing) are not considered.	Reductions in transmission would increase effectiveness of restrictions.
Heterogeneous mixing and travel patterns are not considered.	Heterogeneity could increase or reduce delays in epidemic spread. For example, high transmission among infrequent travelers (e.g., the elderly, children) would make restrictions more effective.
Travel rates and restrictions are based on air-travel volumes alone.	Restrictions would prevent no more than 80% of travel if non–air travel remains unrestricted, which would considerably reduce effect of restrictions.
Seasonal variation in travel and transmissibility are not considered.	Could lead to less or more effective restrictions if arrival of pandemic is in winter/summer.

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Address for correspondence: James Wood, NCIRS, Level 2, Children's Hospital at Westmead, Westmead, New South Wales, Australia 2145; email: jamesw5@chw.edu.au

Emergency Use Authorization (EUA) to Enable Use of Needed Products in Civilian and Military Emergencies, United States

Stuart L. Nightingale,*[1] Joanna M. Prasher,* and Stewart Simonson*[2]

The US Emergency Use Authorization (EUA) is a critical new tool for medical and public health communities and is applicable for both civilian and military use. It fills the need for timely and practical medical treatment under emergency conditions and authorizes use of the best product available for treatment or prevention when the relevant product has not already been approved or approved for this specific use by the US Food and Drug Administration. The need for and genesis of the EUA, its requirements, its broad application to civilian and military populations, and its features of particular importance to physicians and public health officials are detailed.

The Project BioShield Act of 2004 (Public Law 108–276; "the Act"), among other provisions, established the comprehensive Emergency Use Authorization (EUA) program. EUA permits the US Food and Drug Administration (FDA) to approve the emergency use of drugs, devices, and medical products (including diagnostics) that were not previously approved, cleared, or licensed by FDA (hereafter, "unapproved") or the off-label use of approved products in certain well-defined emergency situations. EUA provides physicians and public health officials with an important new tool with wide-reaching implications for medical care under emergency conditions. More detailed information on FDA's policies for authorizing the use of an unapproved medical product or an unapproved use of an approved medical product during a declared emergency can be found in the draft FDA guidance document made available on July 5, 2005 (*1*).

Government Need for EUA Authority

After the events of September 11, 2001, and the anthrax postal attacks <1 month later, the US Department of Health and Human Services (HHS) began developing plans for large-scale off-label use of FDA-approved pharmaceutical products, and in some cases of unapproved products, during a national emergency. This undertaking was especially important at the time because critical components of the biodefense armamentarium were, for various reasons, either unapproved products or approved products whose use as countermeasures was not approved by FDA. At the time, the sole mechanism for making unapproved products available in an emergency was through an Investigational New Drug (IND) protocol or an Investigational Device Exemption.

The medical and public health communities have long recognized that, regardless of how swiftly FDA approves drugs and other medical products, there will always be promising drugs, biologic products (e.g., vaccines, blood products, and biologic therapeutics), and devices (e.g., in vitro diagnostics) that do not have FDA approval (unapproved products) as well as promising off-label uses of drugs, biologic products, and devices that are approved by FDA for other indications. These unapproved or off-label products may be the very best preventive, diagnostic, or therapeutic options available. A physician in practice can prescribe an approved drug for an off-label use or an unapproved drug (subject to state practice of medicine statutes and regulations and FDA policy and legislation) on a patient-by-patient basis. However, large-scale use of unapproved drugs or off-label use of approved drugs, before passage of the Act, could only be carried out under an IND protocol.

IND requirements include Institutional Review Board (IRB) approval of the investigational protocol, documented

*US Department of Health and Human Services, Washington, DC, USA

[1]Current affiliation: National Institutes of Health, Bethesda, Maryland, USA

[2]Current affiliation: Constella Group LLC, Washington, DC, USA

informed consent from all patients describing, among other things, the research purposes of the protocol, substantial record keeping, and patient follow-up requirements. Although an IND mechanism is well-suited for a clinical study and can be used in an emergency situation for an individual patient, the mechanism is ill-suited for situations such as a universal vaccination campaign against a life-threatening infectious disease taking place in the context of a national emergency.

In 1987, FDA issued regulations to establish the Treatment IND as a new regulatory category of INDs (*2*). The purposes, requirements, and implications for physicians of the Treatment IND are described in the medical literature (*3*,*4*). The Treatment IND allows the more extensive use of an investigational product for treatment of life-threatening or serious diseases; however, it did not eliminate the clinical study nature of an IND or the practical aspects that could prove problematic in a national emergency situation. Public health officials would most likely be unable, for example, to comply with the requirements of an IND protocol if smallpox vaccine needed to be administered quickly to a large population during a smallpox epidemic or if an approaching influenza pandemic required widespread distribution and unapproved or off-label use of critical antiviral medications. Experience with attempts to use an IND to offer the licensed anthrax vaccine off-label in a postexposure setting to postal workers possibly exposed to anthrax in the 2001 anthrax postal attack highlighted substantial shortcomings with this approach. While important steps would be taken in good faith to make the IND mechanism work in a national emergency, an alternative was needed: something short of licensure that included specific safety, efficacy, and quality requirements in a manner less administratively burdensome than the IND mechanism. The country needed an emergency mechanism built not on a clinical research model, but on a public health model.

Previous Approaches to Large-Scale Treatment Use of Unapproved Products and Approved Products Off-label

The first widespread use of an IND protocol was in 1971 for admitting narcotics addicts into methadone treatment programs authorized by the US government. Methadone had already long been marketed as a narcotic analgesic and as a cough suppressant, and it was subsequently approved for narcotic maintenance treatment in 1972 (*5*). More than 80,000 patients are estimated to have entered the IND program. This closed distribution system for both the IND and the newly approved use was strictly controlled by FDA and the Drug Enforcement Administration because methadone for narcotic maintenance treatment was only available in programs licensed for this purpose by the government.

During the 1980s, FDA developed several new programs to accommodate the need to make the newest drugs to combat HIV/AIDS and cancer available as quickly as possible (*3*). All of these large programs required IRB review, written informed consent, and reporting that are mandated for other INDs and that could be fulfilled in the context of an ongoing public health situation that, while grave, did not rise to the level of an immediate national security emergency. These programs included the Treatment IND and a new regulatory approach permitting accelerated approval of new drugs.

During the preparations for the Persian Gulf War, the Department of Defense (DoD) determined that several important medical countermeasures would be needed to protect troops in the Gulf. Two of these products were under INDs for these uses at the time: botulinum toxoid to prevent botulism, and pyridostigmine bromide to protect against a chemical nerve agent. Pyridostigmine bromide was already approved by FDA, but the approved indication and dosage formulation were different from those sought for use by DoD. DoD determined that the INDs were needed for force protection in response to specific threats and, on DoD's request, FDA issued an interim final regulation that established a special IND process that included the waiver of informed consent (*6*). This policy evolved on the basis of the danger that individual refusal to take these medications would threaten the well-being of not only that soldier but also others in the unit, thus compromising force protection and the success of the military mission (*7*). This regulation was later rescinded (*8*) and replaced with new legislation for DoD that requires specific presidential approval for waiving informed consent in each military emergency (*9*). The EUA, with its provisions for both military and civilian uses, would later provide an alternative to this special IND process.

For civilian defense, HHS has developed over the past decade a national stockpile of medical countermeasures that could be used in the event of a biological, chemical, radiologic, or nuclear attack. More recently, the national stockpile has been acquiring antiviral drugs, investigational and approved influenza vaccines based on highly pathogenic avian influenza (H5N1) strains, respirators, masks, and other items to prepare the United States to respond to pandemic influenza. Since many of the drugs and other medical products in the national stockpile were either unapproved or were not approved for the countermeasure indication, the Centers for Disease Control and Prevention (CDC) developed protocols for their use under the IND mechanism. As discussed above, however, these protocols would most likely fall short in providing the flexibility needed for effective use in national emergency situations.

Requirements for Granting and Implementing an EUA

The Project BioShield Act of 2004 included new language for section 564 of the Food, Drug and Cosmetic (FD&C) Act that created the EUA in a provision entitled Authorization for Medical Products for Use in Emergencies (*10*). The EUA provides an effective solution to the challenges posed by emergencies involving both civilian and military populations. It addresses the off-label use of FDA-approved products and the use of unapproved products for prevention, treatment, or diagnosis under emergency circumstances. The steps required by the Act before issuance of an EUA are shown in the Table.

Issuance of an EUA is predicated on a Declaration of Emergency that justifies the authorization of the EUA by the secretary of HHS. The secretary may declare such an emergency on the basis of any of the following: 1) the secretary of Homeland Security determines there is a "domestic emergency, or a significant potential for a domestic emergency, involving a heightened risk of attack with a specified biological, chemical, radiologic, or nuclear agent or agents"; 2) the secretary of defense determines that there is a similar emergency or potential emergency threatening military forces; or 3) the secretary of HHS determines that there is a "public health emergency under section 319 of the Public Health Service Act that affects, or has a significant potential to affect, national security, and that involves a specified biological, chemical, radiological, or nuclear agent or agents, or a specified disease or condition that may be attributable to such agent or agents" (*10*). The Act has no requirement that the emergency be the result of a deliberate attack with a CBRN agent to permit use of the EUA. For example, the secretary of HHS could find that an emerging infectious disease or pandemic (such as pandemic influenza) is so serious that it could rise to the level of affecting national security and thus declare a public health emergency under the terms of the Act.

Following the HHS secretary's Declaration of Emergency justifying issuance of the EUA, the FDA commissioner, under delegated authority from the secretary of HHS, may issue an EUA after consultation, to the extent feasible and appropriate given the circumstances of the emergency, with the directors of the National Institutes of Health (NIH) and CDC, if he or she concludes that 1) the agent listed in the emergency declaration can cause a serious or life-threatening disease or condition; 2) on the basis of the totality of scientific evidence available, including data from adequate and well-controlled clinical trials, if available, it is reasonable to believe that the medical product may be effective in diagnosing, treating or preventing this disease or condition or a serious or life-threatening disease or condition caused by another EUA-authorized product or an otherwise approved or licensed product; 3) the known and potential benefits of the medical product, when used to diagnose, prevent, or treat the disease or condition, outweigh the risks, both known and potential; and 4) no adequate, approved, alternative medical product is available.

In addition to these statutory requirements, HHS, through its Office of the Assistant Secretary for Preparedness and Response (formerly the Office of Public Health Emergency Preparedness), has established the Secretary's Emergency Use Authorization Working Group (EUA WG). This is an interagency committee consisting of federal officials with expertise in public health, medicine, law, ethics, and risk communication. It provides recommendations to both the secretary and the FDA commissioner on use of the EUA, as well as facilitating education and communication about the EUA with healthcare professionals and the public.

Although an EUA may not be issued until after an emergency has been declared by the secretary, FDA recognizes that during such exigent circumstances, the time available for the submission and review of an EUA request may be severely limited. Therefore, FDA strongly encourages an entity with a possible candidate product, particularly one at an advanced stage of development, to contact the FDA center responsible for the candidate product even before a determination of an actual or potential emergency is made. The types of information FDA believes are important to allow an assessment of safety and effectiveness of a product and to make an adequate risk-benefit determination to support issuance of an EUA are provided in the FDA draft EUA guidance previously mentioned (*1*). If, before the Declaration of Emergency, FDA believes that a candidate product may meet the criteria for an EUA, the agency may share appropriate information on such product with the secretary's EUA WG.

Table. Actions required before issuance of an EUA*

Step	Required action	Responsible authority
1	Determination of an emergency justifying issuance of an EUA	Secretary of Homeland Security OR Secretary of Defense OR Secretary of Health and Human Services
2	Declaration of emergency	Secretary of Health and Human Services
3	Consultation (to the extent feasible) between the FDA, NIH, and CDC	FDA commissioner, NIH director, CDC director
4	Issuance of an EUA	FDA commissioner (under delegated authority from Secretary of Health and Human Services)

*EUA, Emergency Use Authorization; FDA, US Food and Drug Administration; NIH, National Institutes of Health; CDC, Centers for Disease Control and Prevention.

If the decision is made to issue an EUA, to the extent practicable given the circumstances of the emergency, the FDA commissioner must prescribe certain conditions of use aimed at protecting public health and may prescribe additional conditions for protecting public health. These conditions, and which ones are mandatory, differ depending on whether the EUA authorizes use of an unapproved product or authorizes an off-label use of an FDA-approved product. However, certain basic provisions must be met under all cases, examples of which are described below (a more detailed description of conditions of use that may be applied can be found in the draft FDA guidance [*1*]).

For example, to the extent practicable given the circumstances of the emergency, both healthcare providers and their patients must be made aware that the product has been authorized for emergency use, must know the "significant known and potential benefits and risks" of emergency use of the product and extent to which such benefits and risks are unknown, and must be informed of any alternatives that may be available and their benefits and risks. Additionally, as a general rule, persons must be made aware of their right to refuse the product (or to refuse it for their children or others without the capacity to consent) and of the potential consequences, if any, of this choice. An exception to this rule is that the president, as commander in chief, can waive military personnel's right to refuse this product. If the right is not specifically waived by the president for a particular product given under EUA, military personnel have the same right to refuse as civilians. FDA expects that such information will be disseminated to healthcare providers and the general public in the most effective and expeditious way possible, including use of informational leaflets, the media, Internet, videos, and direct communications from public health officials.

During administration of an EUA product, a system would be developed to collect and analyze safety and efficacy information on unapproved products, and such a system may also be developed for unapproved uses of approved products. Adverse events arising from use of the product would be carefully monitored and reported. The FDA commissioner would periodically review the safety and efficacy data collected on EUA products and could revoke the EUA at any time if the criteria for the EUA are no longer met or to protect public health and safety. For unapproved products, the commissioner may choose to designate the persons or entities that may distribute and administer the product for emergency use. For example, the commissioner may choose either to route products under an EUA to central dispensing sites or, in cases where the transportation and congregation of large populations are either dangerous or impractical, allow for distribution of required medications and appropriate product information by postal workers or others. Unless previously revoked or renewed, an EUA will expire 1 year after the Declaration of Emergency.

Implications of the EUA for Physicians

Although similarities exist, use of a product under an EUA is substantially different from use of a product under an IND protocol. For example, EUA products do not require the detailed, formal, informed-consent process used for human research study participants. However, to the extent practicable given the circumstances of the emergency, prospective patients will always be informed about the opportunity to accept or refuse an EUA product (except for those cases noted above in which the president has specifically waived this right for military personnel) and be given all the information necessary to make this informed choice, as they would for any product offered to them by their healthcare provider. Other unique features of medical product distribution under an EUA include the fact that requirements for the distribution and administration of a EUA product will be determined by the FDA commissioner, in consultation with the directors of CDC and NIH, on a case-by-case basis for each EUA requested. In addition, EUAs do not have to be reviewed by an IRB when they are used for public health purposes. Also, there is no requirement that would prevent EUA products from being dispensed without a physician's prescription; thus, in a national emergency, prescription products could be provided by a nonlicensed provider or any distribution method or location approved by the FDA commissioner in issuing the EUA.

Concerns have been raised about the liability and compensation protections associated with potential use of a medical product under an EUA. The Public Readiness and Emergency Preparedness Act of 2005 (Pubic Law 109–148), provides immunity from liability claims arising from administration and use of covered countermeasures to involved manufacturers, distributors, program planners, and qualified persons (with the exception of claims arising from willful misconduct). Covered countermeasures are those that address a disease or condition that the HHS secretary has determined poses a public health emergency or a credible risk of causing a public health emergency in the future. The first such declaration supporting liability protection was made by the secretary in February 2007 regarding vaccines to address pandemic influenza (*11*). This same coverage could be used for other medical countermeasures in the future, including those that would be used under an EUA.

Although we describe recent legislation in the United States that enables emergency use of products not yet approved by the FDA, or approved only for uses or administration not suitable for emergency situations, we are aware that some other countries have developed procedures

to permit such use. We are also aware that various other countries' national legislation would not restrict such off-label use, or even use of unapproved products, in emergency situations. We believe that sharing general information on potential mechanisms for addressing these issues can help all countries better prepare for, and respond to, emergencies of all types. Most importantly, sharing experiences in the authorization and use of particular products to address emergency needs will be especially helpful to other countries as they identify countermeasures for their own stockpiles.

EUA for Anthrax Vaccine Adsorbed (AVA)

The first use of the EUA authority was in 2005. It occurred in response to a unique set of circumstances, but nonetheless stands as an example of an effective public health response to a need for large-scale use of a medical countermeasure to a biologic agent. Since 1998, to protect against the threat of anthrax attack, the armed forces have vaccinated a substantial number of their members with AVA, a vaccine licensed since 1970 but not originally contemplated as a biowarfare or bioterrorism countermeasure. The program has had detractors and has been the subject of litigation. In late 2004, a federal court issued an injunction against the DoD program on the grounds that the FDA should have obtained public comments before issuing a determination confirming that the AVA license included use for prevention of inhalation anthrax. The court decision effectively deemed use of AVA to prevent inhalation anthrax an unapproved use of an approved drug.

While awaiting the conclusion of the public comment process, DoD, to address what it considered to be the adverse effect of the injunction on military readiness, asked for an EUA to allow a continuation of military vaccinations against anthrax. Then Deputy Defense Secretary Paul Wolfowitz (with assigned authority from the secretary of defense) determined on December 22, 2004, pursuant to the Act, that there was a significant potential for a military emergency involving anthrax and requested that an EUA be issued for AVA. Then–HHS Secretary Tommy G. Thompson issued a Declaration of Emergency on January 14, 2005 (*12*). On the basis of this declaration and having concluded that the criteria for issuance of an EUA were met, then–Acting FDA Commissioner Lester Crawford, in consultation with the directors of NIH and CDC, issued an EUA for AVA on January 27, 2005 (*13*). In this instance, therefore, the time from request for an EUA to issuance of the EUA was 5 weeks. The timelines for FDA review and action on a request for consideration for an EUA will depend on the product profile; the existence, if any, of pending pre-EUA applications for the product; the nature of the emergency; and other relevant factors. Although the time required for FDA action will vary, FDA recognizes that it is likely that, in an emergency situation that is occurring or believed imminent, a request for consideration for an EUA will be acted upon within a matter of hours or days.

Importantly, the EUA issued by Dr. Crawford required DoD to inform military members that they had an option to refuse the vaccine and that no adverse action would be taken against those who declined the vaccine under the EUA. The issuance of this EUA cleared the way for DoD to resume anthrax vaccinations to protect military personnel assigned to certain higher threat areas. This first use of the EUA authority illustrates its important statutory purpose: FDA determined that anthrax vaccine was the best available medical countermeasure to the potential military emergency posed by the risk for attack with anthrax and allowed DoD to use it.

The EUA for AVA was originally issued for 6 months on the request of DoD. Under the Act, an EUA can be extended within the duration of the Declaration of Emergency if the criteria under Section 564(c) of the FD&C Act for issuance of such authorization are still met. On July 22, 2005, the then–FDA commissioner extended the EUA for the duration of the Declaration of Emergency, which terminated on January 14, 2006. During the period of the EUA, more than 100,000 anthrax vaccinations were given. The EUA was allowed to expire on January 14 because FDA, on December 19, 2005, issued a final order concluding that AVA is safe and effective for its labeled indication, to protect persons at high risk for anthrax disease. This action permitted DoD to resume vaccination with AVA for its licensed indication, and an EUA was no longer required.

Conclusions

EUA is a critical new tool for the medical and public health communities and is applicable for both civilian and military use. It fills the need for timely and practical medical treatment when the relevant product has not already been approved or approved for this specific use by the FDA. An understanding of this new product category and its implementation is important to those who will be on the frontlines providing direct care, as well as to those who will be managing mass care situations.

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Dr Nightingale recently retired from HHS, where he was deputy assistant secretary for preparedness and response. Currently, he is a consultant (contractor) to the National Institutes of Health's Office of the Director and the National Institute for Allergy and Infectious Diseases on issues related to domestic and international bioterrorism, emerging infectious diseases, oversight of dual use research, and EUAs for medical countermeasures.

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Address for correspondence: Stuart Nightingale, Office of Biotechnology Activities, National Institutes of Health, 6705 Rockledge Dr, Suite 750, Bethesda, MD 20892-7985, USA; email: nightins@od.nih.gov

Influenza Pandemics in Singapore, a Tropical, Globally Connected City

Vernon J. Lee,*†‡ Mark I. Chen,* Siew Pang Chan,* Chia Siong Wong,* Jeffery Cutter,§ Kee Tai Goh,§ and Paul Anath Tambyah¶#

Tropical cities such as Singapore do not have well-defined influenza seasons but have not been spared from influenza pandemics. The 1918 epidemic in Singapore, which was then already a major global trading hub, occurred in 2 waves, June–July, and October–November, and resulted in ≥2,870 deaths. The excess mortality rate was higher than that for industrialized nations in the Northern Hemisphere but lower than that for less industrialized countries in Asia and Africa. The 1957 epidemic occurred in May and resulted in widespread illness. The 1968 epidemic occurred in August and lasted a few weeks, again with widespread illness. Tropical cities may be affected early in a pandemic and have higher mortality rates. With the increase in travel and trade, a future pandemic may reach a globally connected city early and spread worldwide. Preparedness and surveillance plans must be developed to include the megacities of the tropical world.

Influenza has had a substantial effect worldwide. The 3 influenza pandemics of the 20th century (1918–9, Spanish Flu; 1957–8, Asian Flu; and 1968, Hong Kong Flu) resulted in 40 million, 2 million, and 1 million deaths, respectively (*1*,*2*). Their social, cultural, and economic effect has been best described in North America and Western Europe (*3*).

Although tropical countries such as Singapore do not have as well-defined influenza seasons as temperate regions, they are not spared from the effects of influenza (*4*). Each year, 20% of Singapore's population is estimated to be clinically infected from seasonal influenza (*5*). Deaths caused by influenza in Singapore over the past decade were ≈14.8 per 100,000 person-years, which is comparable to deaths caused by this disease in the temperate United States and subtropical Hong Kong Special Administrative Region, People's Republic of China (*6*). However, the effect of pandemic influenza in tropical cities has not been well described. This study aims to describe the effect of these pandemics on Singapore, a global trading city throughout the 20th century. The lessons learned from the effect and management of previous pandemics may have implications for pandemic planning in tropical global trading cities.

Methods

To determine the effect of influenza on mortality rates during the pandemic years, we obtained monthly mortality rate data from various official sources in Singapore. For the years surrounding the 1918 pandemic, data were obtained from the Annual Departmental Reports of the Straits Settlements (the British colonies that included Singapore, Penang, Malacca, and Labuan; the last 3 are now part of Malaysia), and from the Registry of Births and Deaths, Singapore. For the years surrounding the 1957 and 1968 pandemics, data were obtained from the Registry of Births and Deaths, Singapore. These were the only official government departments responsible in the respective years for the collection and verification of these statistics.

Because tropical countries do not have well-defined influenza seasons, methods for the analysis of excess deaths in temperate countries such as that used by Serfling et al. (*7*) may not be appropriate because the assumption of influenza seasons in distinct, regular waves may not be valid. We have thus elected to use direct statistical analysis of data for the 2 years before and after the pandemic year to form a regression line with 95% confidence intervals. Deaths for each month were then compared with the regression line. Months for which the mortality rate exceeded the 95% confidence intervals were considered as those with ex-

*Tan Tock Seng Hospital, Singapore; †National Healthcare Group, Singapore; ‡Ministry of Defence, Singapore; §Ministry of Health, Singapore; ¶National University of Singapore, Singapore; and #National University Hospital, Singapore

cess deaths, with the excess represented as the actual mortality rate minus the predicted mortality rate.

To provide another perspective of possible excess deaths for comparison, we used another method described by Murray et al. for 1918, with a simpler equation to estimate excess deaths (*8*). For 1918, death rates during the 3-year pandemic window were compared with those in surrounding years, i.e., the average mortality rate for the 3 years before and after were subtracted from the mortality rate during the 3-year pandemic window.

In addition to statistical analyses, we conducted a detailed search of peer-reviewed journal articles, government reports, and press articles for the 3 pandemics. Search results provided comparisons of the mortality rates in other countries and an overview of the public health issues and interventions conducted in Singapore and how they compared with those of other countries and current recommendations.

Results

The 1918 Pandemic in Singapore

The 1918 Straits Settlements Annual Report described an influenza epidemic in June and July that was relatively mild, with a high illness rate but a low mortality rate, that peaked during the week ending July 6 (*9*). A second intense wave occurred in October and November, leading to frequent pneumonia and a high mortality rate. It peaked during the week ending October 26, with 97.6 deaths per million population (*10*).

The 1918 Annual Report indicated 844 recorded influenza deaths. However, the Straits Settlements' overall annual mortality rate was "43.85 per thousand in 1918 when the influenza epidemic struck the country" (*10*). This is in contrast to the immediate prepandemic and postpandemic years from 1915 to 1921, when mortality rates ranged from 29/1,000 to 37/1,000 population. The excess mortality rate within the Straits Settlements in 1918 was therefore 11.3/1,000 (9,435/827,719).

Figure 1 shows that the excess mortality rate of the epidemic in Singapore alone, as calculated by our method, was 7.76 per 1,000 (2,870/369,800) during May–June and October–November 1918. Using the formula of Murray et al. (*8*), we calculated the excess mortality rate for Singapore during the pandemic years of 1918–20 to be 1.80% (18/1,000, or 6,656 deaths).

The excess mortality rate for Singapore during the 1918 pandemic years was higher than rates for most industrialized countries such as the United States and those for western Europe (Table), but lower than rates for African and Asian countries such Kenya, South Africa, India, and the Philippines. The excess mortality rate of 1.80% for Singapore was higher than the global average rate of 1.06% and higher than the rate for other Asian countries such as Taiwan (1.44%) (*8*).

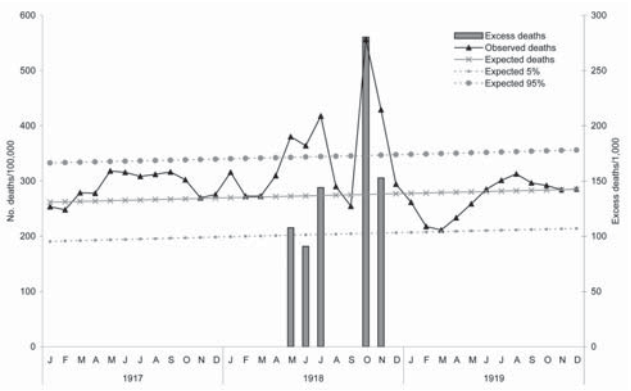

Figure 1. All-cause monthly mortality rates, Singapore, 1917–1919.

To reduce the effect of the pandemic, the government used available evidence to institute a series of preventive measures. The government and physicians advised infected persons to isolate themselves and seek treatment, to disinfect the floors of public premises daily, and, during the second wave of the outbreak, to avoid crowded places (*16*). Suggestions were also made to restrict or prohibit visiting of hospitalized patients, and schools were closed for a week at the peak of the second wave (*16*). Recommended prophylactic measures included reducing the amount of fatigue and maximizing ventilation.

By the end of November 1918, the epidemic was over in Singapore, although the media still reported the disease in Indonesia, New Zealand, South Africa, Japan, and other regions. There were no local reports or evidence of a third wave similar to that in temperate countries in early 1919 (*17*).

The 1957 Pandemic in Singapore

The media declared the 1957 pandemic as the "worst ever in colony [Singapore] history" (*18*). The outbreak was first recognized at the end of April and early May and was purported to have spread through Hong Kong from its origins in northern Asia (*19*). By May 5, the outbreak had become an epidemic, reaching its peak in mid-May and tapering off by the end of the month (*20*). In May, 77,211 (47.6%) of 162,093 patients who came to government and city council clinics were treated for influenza; 326 required hospital admission, and 28 deaths from influenza were recorded (22 from pneumonia and 6 from cardiac complications) (*20*). On the basis of monthly mortality rate reports (Figure 2), an excess mortality rate of 0.47/1,000 occurred in May 1957. This represented 680 deaths in a population of 1,445,900. There was another small peak of excess deaths in October 1958, although this was only slightly above the baseline value.

Table. Estimated deaths and mortality rates due to influenza during the 1918–1920 influenza pandemic

Country	No. deaths (in 1,000s)	Mortality rate (per 1,000), %	References
United States	402–675	3.9–6.5	(*8*,*11*–*13*)
Canada	50.0–51.0	6.1–6.3	(*8*,*12*)
Denmark	6.02–12.4	2.0–4.1	(*8*,*12*)
England	116–200	3.4–5.8	(*8*,*12*)
Spain	257–311	12.3–14.9	(*8*,*12*)
Portugal	59.0–159	9.8–26.4	(*8*,*12*)
India	185	6.1–43.9	(*8*,*12*)
Japan	368–517	6.7–9.4	(*8*,*12*)
Ceylon (Sri Lanka)	51.0–91.6	10.0–17.9	(*8*,*12*,*14*)
Taiwan	25.4–52.8	6.9–14.4	(*8*,*12*)
The Philippines	81.0–288	8.0–28.4	(*8*,*12*)
Argentina	10.2–46.0	1.2–5.4	(*8*,*12*)
Australia	14.5–15.4	2.7–2.9	(*8*,*12*)
Kenya	104–150	40–57.8	(*8*)
South Africa	300	44.3	(*8*)
British Honduras (Belize)	1.01–2.00	2.3–4.6	(*15*)
Trinidad and Tobago	0.30–1.00	0.1–0.2	(*15*)
Singapore	2.87–6.66	7.8–18.0	This report

During the 1957 epidemic, the government focused on public health measures, including closure of schools for almost 2 weeks because of illness and absenteeism. The public was advised to keep away from crowded places (*20*), and the slogan "no movement of persons – no spread of influenza" was professed (*21*). At healthcare facilities, elective surgery was minimized to release staff to manage the epidemic. School health clinics, maternal and child health clinics, and voluntary clinics were set up as influenza treatment centers (*20*). Although no port quarantine measures were required by law, the airport health officer checked outward-bound passengers for airlines upon request. Similarly, 1 shipping line screened all passengers boarding their ships, and those who failed screening were denied embarkation (*20*).

The 1968 Pandemic in Singapore

The 1968 pandemic was the mildest of the 3 pandemics; the epidemic in Singapore occurred in early August and lasted for a few weeks. The virus was believed to have spread from a major outbreak in Hong Kong (*22*).

The outbreak in Singapore peaked August 16–25. Attendance at outpatient dispensaries increased over a 2-week period, and at the peak daily attendance increased 65% from 6,052 to 9,966 (*23*). On the basis of monthly mortality rates (Figure 3), the excess mortality rate was 0.27/1,000 (543/2,012,000) during August and September 1968. Excess deaths peaked again in May and June 1970, which mirrored a possible second pandemic wave, as reported worldwide in 1969–70, although the lower second wave excess mortality rate was similar to rates in the Americas and different from rates in Europe and Asia (*24*). The excess mortality rate for 1970 was 0.15/1,000 (309/2,074,500).

The 1968 epidemic caused substantial illness and absenteeism from work. However, because of the relatively mild and short epidemic, no substantial measures were adopted. The Ministries of Education and Health considered the closure of schools but decided against it because of the waning of the epidemic (*25*).

Discussion

Excess mortality rates vary according to the method used for calculation and sources of data, facts that reiterate the difficulty of conducting historical estimates. Nevertheless, the estimated number of influenza deaths (2,870–6,656) in Singapore in 1918 exceeded the official report of 844 influenza deaths. The 1918 Annual Report admitted that the latter number poorly represented actual deaths, which it estimated more accurately at 3,500 (*9*). The 1921 Annual Report added that many deaths reported as pneumonia were due primarily to influenza (*10*). This showed that tropical Singapore had mortality rates comparable to or exceeding those of temperate regions (Table). Similarly, the calculated excess deaths of 680 in 1957 exceeded the 28 recorded influenza deaths.

The excess mortality rate for Singapore (Table) supports the hypothesis that income levels and development were negatively correlated with influenza mortality rates (*8*,*26*) because Singapore was less industrialized than many industrialized Western cities and nations in the early 20th century. However, Singapore, as a main trading city, was relatively more industrialized with a proportionately smaller rural population, and thus had lower mortality rates than did neighboring countries such as India and the Philippines (Table). Even in Singapore, attack rates were lower for Europeans and Asians with higher socioeconomic sta-

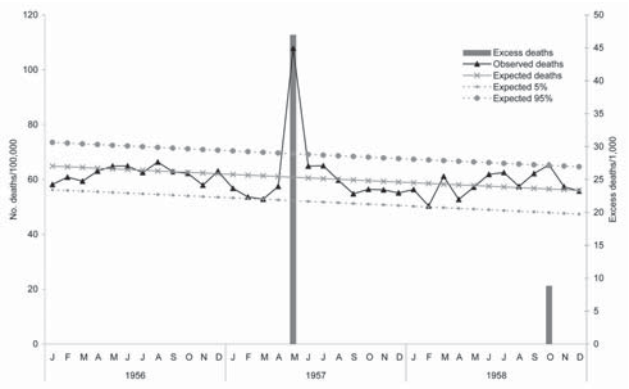

Figure 2. All-cause monthly mortality rates, Singapore, 1956–1958.

tus (6.0%–20.4%) than for persons with a lower socioeconomic status (29.0%–29.8%), which suggested that socioeconomic status had a possible role in disease transmission (*19*,*27*). Another possible explanation is that those who were more educated were also more receptive to public health messages, which reduced disease transmission.

Using the formula of Murray et al. for the 1918–20 pandemic, we determined that the excess mortality rate for Singapore was higher than the global average rate. Because the Singapore epidemic occurred early in the global pandemic, this finding corroborates the suggestion that early epidemic centers experienced higher mortality rates (*8*). This is also evident when one compares the mortality rates for tropical countries such as Ceylon (present day Sri Lanka) and Singapore with rates for tropical Caribbean islands (Table). During the second wave of the pandemic in Spain in October 1918, Asian nations such as Ceylon and Singapore also reported similar epidemic recrudescence in early October (*28*). By the end of the second wave of the pandemic in Singapore, there were still reports of influenza in Malaysia, Indonesia, New Zealand, and Japan (*9*,*29*). This finding also suggests that nations are at high risk of acquiring early infection and could act as sentinels for the next pandemic.

The effect of all 3 pandemics was felt across Singapore. However, reported overall mortality rates of 43.85/1,000 in 1918 were comparable to "46.46 per thousand in 1911, a very malarious year" (*10*). Deaths during the first epidemic wave were initially attributed to malaria (*30*). The 1918 pandemic also had a variable effect in US possessions in tropical regions (*3*). The early effect from the 1918 Singapore epidemic may not have been noticeable because of the nonseasonal nature of influenza in the tropics (*4*) or because of the high background mortality rates from infectious diseases and other causes in Singapore. Although excess deaths in 1918 were substantially higher than excess deaths in 1957, the relative change in mortality rates was similar; peak monthly mortality rates were twice baseline mortality rates for both periods (Figures 1, 2). The 1918 baseline mortality rate was 4× higher than the 1957 rate, and the decrease in the baseline mortality rate was largely due to improved socioeconomic conditions and control of infectious diseases such as malaria. With the low baseline mortality rate for modern cities, the effect of a pandemic, however mild, may be noticed (the Singapore media declared the 1957 pandemic as the worst). Although studies suggest that pandemic mortality rates will be higher for industrialized countries (*8*,*26*), if a pandemic were to first appear in less industrialized regions with high baseline mortality rates, the pandemic might be missed or dismissed as yet another spike of endemic infectious diseases during the initial epidemic phase until deaths increased.

Apart from illness and death, subpopulations were also severely affected by the pandemics. In 1957, the closure of 670 schools affected 262,000 students who required alternative care and education. Commercial firms reported staff absenteeism of 10%–30% (*31*). Clinics were frequently overwhelmed, and available healthcare workers were recalled to cope with the increase in influenza patients. However, healthcare workers were at high risk for infection (*14*,*31*). In 1918, 12 (63%) of 19 nurses at the Singapore General Hospital were concurrently ill (*32*); in 1957, 25% of the nursing staff in Taiping (a Malaysian town) were ill (*33*). Healthcare workers were stressed as they coped with personal illness and increased numbers of patients.

Although more is now known about influenza pathology and epidemiology, in 1918, influenza was correctly reported as being highly infectious and spread by breathing, coughing, and spitting, and having an incubation period "from a few hours to three days" (*34*). Even with the knowledge gap, measures such as respiratory hygiene, social distancing, and disinfection were promoted (*16*,*19*). In the recent World Health Organization recommendations for pandemic influenza, respiratory hygiene has been encouraged as a routine preventive measure (*35*). Social distancing and disinfection may also be considered to reduce its effect, depending on severity and transmission of disease, to reduce its effect, although definitive evidence is

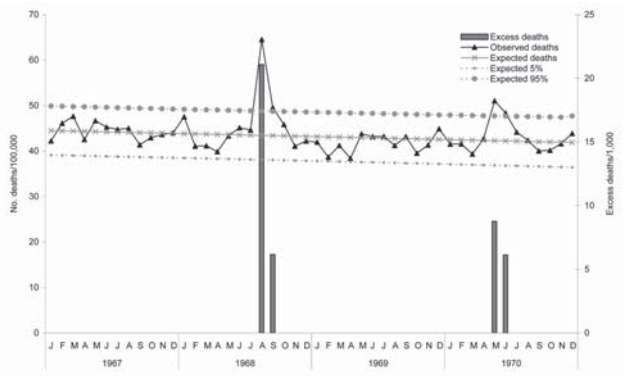

Figure 3. All-cause monthly mortality rates, Singapore, 1967–1970.

lacking (*35*). The effect of school closures remains unclear. Ferguson et al. suggested that closure of schools does not substantially reduce overall attack rates but does reduce peak attack rates (*36*). Germann et al. suggested that school closures may be effective if conducted early in pandemics with low reproductive numbers (low R_o values) (*37*). However, interventions such as travel restrictions and border controls have been shown to be not feasible (*36*). Although some of these measures may reduce illness and death, they have to be weighed against productivity losses and socioeconomic effects of the interventions.

With the increase in travel and trade, a future pandemic may reach a globally connected city before preparedness plans can be fully activated. The 1918 pandemic is thought to have originated early in the year and had spread to Singapore by June. Another globally connected city, New York City, also showed an early wave in February–April 1918 (*38*). The 1957 and 1968 epidemics arrived weeks after their suspected origins in northern Asia because of travel from Hong Kong, another globally connected city (*21*,*23*). These type of cities are also the focal point of spread, as shown by the spread of influenza from Singapore to India in 1957 (*39*).

Mortality rates suggest that the 1918 epidemic in Singapore may have occurred in May, which is earlier than in official reports (Figure 1). This finding suggests the possibility of late recognition. Delayed recognition must be considered even in this modern age. In 2003, the severe acute respiratory syndrome (SARS) epidemic reached Singapore within weeks of its appearance in the southern part of the People's Republic of China but remained undetected. Two of the 20th-century influenza pandemics and the SARS epidemic are believed to have originated from farms in eastern Asia. SARS was first detected in Foshan, quickly spread to Guangzhou City (a major regional trading hub), to Hong Kong, and then to the rest of the world. A global surveillance effort is therefore critical to enable prompt activation of pandemic plans. This effort should include frontline surveillance of farms in eastern Asia and secondary surveillance of major Asian cities.

Trading hubs may be affected early in the course of a pandemic and show higher mortality rates. The megacities of Asia, Africa, and Latin America are now extensively involved in global trade and travel networks and are more likely to be affected by a pandemic. However, influenza is a difficult surveillance target, with an accuracy of clinical diagnosis in 1968 of only ≈66% (*40*). A good laboratory surveillance network in major cities is therefore critical to enable accurate diagnosis and virus identification.

This study has some limitations. Mortality rate data in Singapore, although of good quality because of the small size of the country, were available only from limited sources. We have attempted to use estimates from other government agencies such as the health department and ministry of health. Weekly data would have provided better information, but quality data were available in the press only for certain weeks, which we have presented.

Globally connected cities will be especially vulnerable to a future pandemic, and preparedness plans must be developed to include the megacities of the tropical world. The 20th-century pandemics swept through Singapore within 4 weeks; future plans must include such a possibility over a similarly short duration. Public health measures such as surveillance and preparedness plans must be formulated to slow the spread of a pandemic and mitigate its effects.

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Dr Lee is a preventive medicine physician with the Singapore Ministry of Defence, the Communicable Disease Centre, Singapore, and the National Healthcare Group, Singapore. His research interests include emerging infectious diseases preparedness, health economics, and health services research.

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Address for correspondence: Vernon J. Lee, Block 802, Communicable Disease Centre, Moulmein Rd, Singapore 308433; email: vernonljm@hotmail.com

Rocky Mountain Spotted Fever, Colombia

Marylin Hidalgo,* Leonora Orejuela,† Patricia Fuya,* Pilar Carrillo,‡ Jorge Hernandez,§ Edgar Parra,* Colette Keng,¶ Melissa Small,¶ Juan P. Olano,¶ Donald Bouyer,¶ Elizabeth Castaneda,* David Walker,¶ and Gustavo Valbuena†¶

We investigated 2 fatal cases of Rocky Mountain spotted fever that occurred in 2003 and 2004 near the same locality in Colombia where the disease was first reported in the 1930s. A retrospective serosurvey of febrile patients showed that >21% of the serum samples had antibodies against spotted fever group rickettsiae.

Between July 1934 and August 1936, sixty-five cases of Rocky Mountain spotted fever (RMSF), 62 of them fatal, were reported from Tobia, Colombia (*1*). No reports of this disease (known locally as Fiebre de Tobia) have been produced from Colombia since, and currently RMSF is generally not included in the differential diagnoses of febrile syndromes.

We recently confirmed RMSF as the cause of death for 2 patients by PCR (*2*,*3*), sequencing, immunohistochemical tests (*4*), and culture (*5*) (Table 1). The first patient was a 32-year-old pregnant woman (26 weeks), who had abdominal pain, headache, and fever in December 2003; pharyngitis was diagnosed, and she received amoxicillin with no improvement. A cutaneous macular rash, hepatomegaly, hyperbilirubinemia, leukocytosis, and thrombocytopenia (50,000/μL) subsequently developed. She then experienced respiratory failure and died. One of her relatives, as well as 2 dogs, had died a few days earlier with similar symptoms. Another sick dog rapidly recovered after receiving doxycycline.

The second patient was a 31-year-old previously healthy man who went to the local hospital in May 2004 with fever and severe headache; dengue was diagnosed clinically. Three days later, he became stuporous and was admitted to the hospital. Within a short period, seizures developed and he became comatose. He died a few hours later.

The 2 patients lived near the towns of Villeta and Tobia, Cundinamarca, Colombia. The histopathologic findings of both patients were similar and consisted of vascular congestion; interstitial edema; frequent nonoccluding thrombi (mainly in the lungs); and multiple foci of perivascular lymphocytic and monocytic infiltration in all viscera, including the brain. The lungs showed marked interstitial inflammatory infiltrates. Immunohistochemical analysis showed rickettsiae in the microvascular endothelium of all studied organs, including brain, liver, spleen, and lungs of both patients (Figure).

Several weeks after these events, we collected and identified adult male and female ticks from the farms and surroundings where the patients had lived (Table 1). We found ticks of the species *Amblyomma cajennense*, a known vector of spotted fever group rickettsioses in Latin America (*6*–*9*), and *Rhipicephalus sanguineus*, recently documented as a vector for *Rickettsia rickettsii* (*10*).

To begin to clarify the magnitude of spotted fever group rickettsioses as a public health problem in Colombia, we tested the following samples for spotted fever group rickettsiae by immunofluorescence assay (IFA) (*11*): 1) 64 serum samples from a national Colombian surveillance system (2001–2004) that studies malaria, dengue, and yellow fever (Instituto Nacional de Salud, Colombia); and 2) 96 serum samples from a regional (the state where the reported patients lived) surveillance system (2000–2001) for dengue (Secretaria de Salud de Cundinamarca, Colombia). Serum samples showing distinctly fluorescent rickettsiae at a ≥1:64 dilution were considered positive. We found immunoglobulin G (IgG) and IgM antibodies against spotted fever group rickettsiae (*R. rickettsii* was used as antigen) but not against typhus group rickettsiae (*R. typhi* was used as antigen) (Table 2). These data suggest that spotted fever group rickettsioses may be a frequent cause of febrile illnesses, not only in the state where the reported patients lived but also in various other regions of Colombia. Since there is strong cross-reactivity among rickettsial species when IFA is used as an antibody-detection technique, other spotted fever group rickettsiae, including those recently described

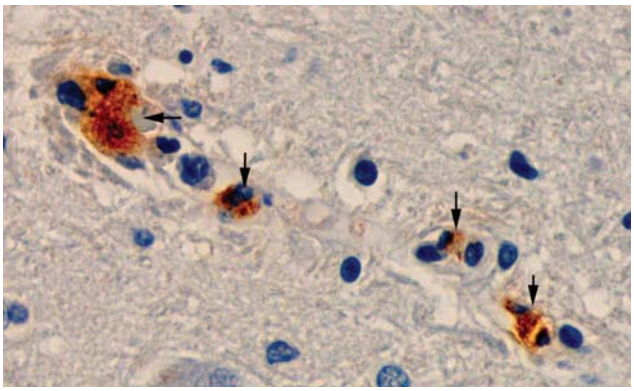

Figure. Immunohistochemical analysis shows the presence of spotted fever group rickettsiae (brown) in vessels of brain of a patient with fatal Rocky Mountain spotted fever (magnification 400).

*Instituto Nacional de Salud, Bogota, Colombia; †School of Medicine at Universidad de Los Andes, Bogota, Colombia; ‡Secretaria de Salud de Cundinamarca, Colombia; §Hospital Salazar, Villeta, Cundinamarca, Colombia; and ¶University of Texas Medical Branch, Galveston, Texas, USA

Table 1. Rocky Mountain spotted fever patients and findings, Colombia, 2003–2004

Patient	Confirmatory methods						Adult ticks collected in area where patients lived
	PCR		Sequence	IHA*	Animal inoculation	Culture	
1	Genes	Primers	Homology with *Rickettsia rickettsii*	Positive with rabbit anti–spotted fever group rickettsial antibody	Not done	Not done	15 *Amblyomma cajennense,* 184 *Rhipicephalus sanguineus,* 7 *Anocentor nitens,* and 8 *Amblyomma* spp.
	17-kDa	17kD1/2	100% (Sheila Smith)				
2	Genes	Primers	Homology with *R. rickettsii*	Positive with rabbit anti–spotted fever group rickettsial antibody	24 h and 48 h after fever onset, 2 guinea pigs were euthanized for culture and PCR analysis of spleens	Vero cells with cytopathic changes after 1 week	36 *A. cajennense,* 13 *R. sanguineus,* and 38 *Boophilus microplus*
	gltA	CS78/323	99% (Bitterroot and others)				
	gltA	CS5/6	94% (Bitterroot)				
	OmpA	190.70/701	98% (strain 1995HO2 and others)				
	OmpB	rOmpB.20-2788	98% (GenBank accession no. X16353.1)				

*Immunohistochemical analysis.

in Latin America (*R. parkeri* and *R. felis*) could explain the assay results (*12,13*). Furthermore, most of these patients received a clinical diagnosis of dengue, an endemic disease in Colombia that appears to have become an umbrella diagnosis under which other diseases are assigned. A similar situation was recently described in Mexico (*14*).

RMSF in Colombia is seldom considered in the differential diagnosis for febrile disease; possible causes include the lack of an adequate diagnostic infrastructure and the invisibility of tick- and fleaborne infectious diseases in most medical curricula. The problem is further compounded by the presence of numerous agents (many transmitted by arthropod vectors) that produce nonspecific febrile syndromes during the early stages of the disease. Most of those agents are viruses that, unlike rickettsiae, have no specific treatment; thus, physicians might not feel compelled to use antimicrobial agents. Given the lack of appropriate and inexpensive diagnostic tests that are useful in the acute stage and that can be implemented in small rural hospitals, the best diagnostic tool available to healthcare personnel is clinical suspicion based on knowledge of the clinical manifestations (*15*), ecology, and epidemiology of rickettsioses. Physicians in areas where RMSF is endemic should consider prescribing a course of empirical treatment with doxycycline in patients who have high fever, severe headache, and myalgia, even in the absence of rash or history of tick bite, as both are frequently absent in RMSF. Such a treatment will not harm a patient with dengue or other viral infections and is likely to save the life of a patient infected with *R. rickettsii*.

Acknowledgments

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Ms Hidalgo is a research scientist at Instituto Nacional de Salud, Bogota, Colombia. She is investigating the epidemiology of rickettsial diseases in Colombia as part of her graduate training for the PhD degree.

Table 2.Titers of antibodies to spotted fever group rickettsiae (antigen: *Rickettsia rickettsii)* by indirect immunofluorescence antibody assay*

Surveillance program†	No. tested	IgG			IgM				States of origin
		No. (%) positive	Titer	n	No. (%) positive	Titer	n	No. also positive for IgG	
National	64	3 (4.7)	128	1	1 (1.5)	512	1	0	Santander, Guaviare, Caldas
			256	2					
Regional	96	21 (21.9)	64	3	20 (20.8)	64	3		Cundinamarca
			128	10		128	4		
			256	4		256	9	10	
			512	3		512	3		
			1,024	1		1,024	1		

*Ig, immunoglobulin.
†Acute Febrile Disease Surveillance Programs.

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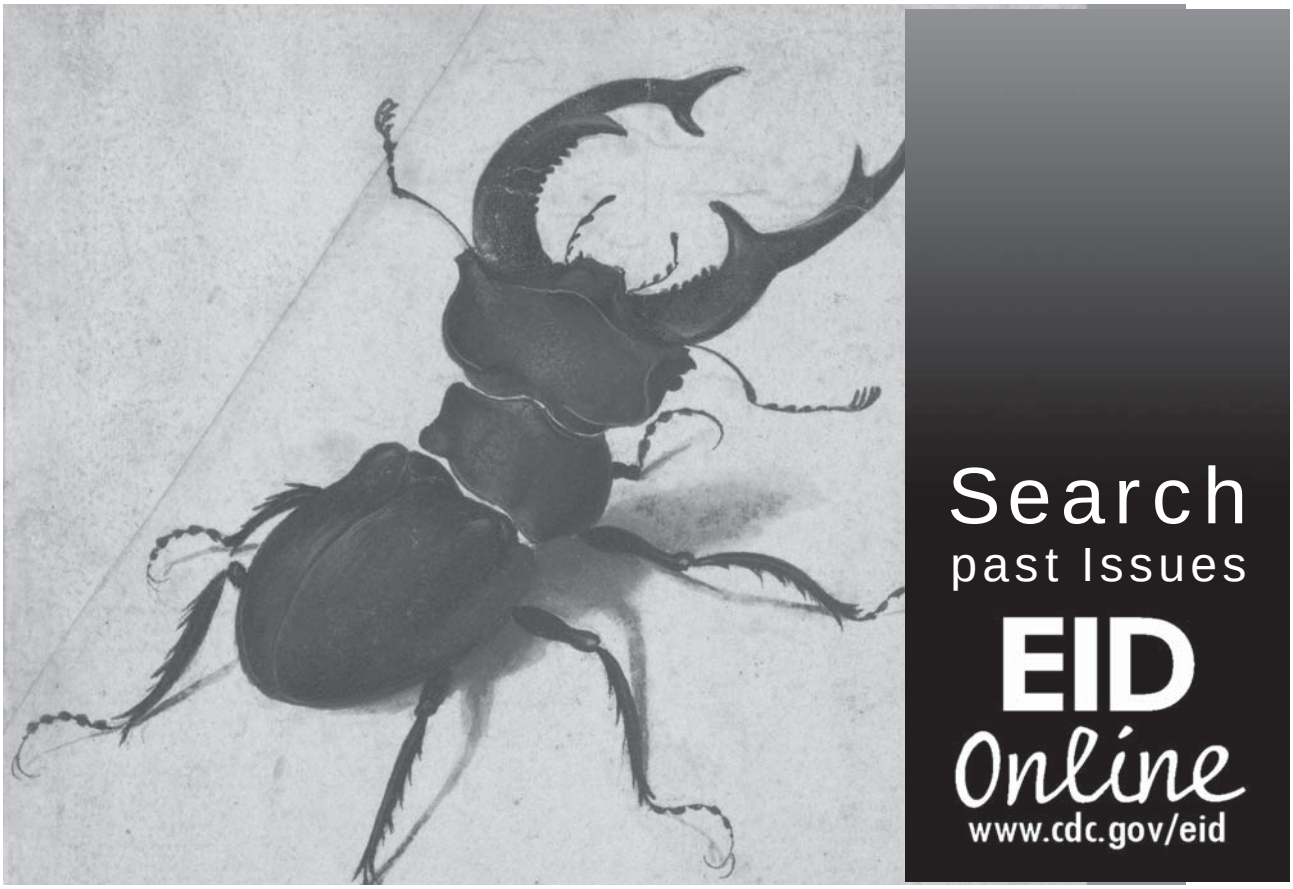
Address for correspondence: Gustavo Valbuena, Department of Pathology, The University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555-0609, USA; email: gvalbuen@utmb.edu



Human Influenza A (H5N1) Cases, Urban Areas of People's Republic of China, 2005–2006

Hongjie Yu,*[1] Zijian Feng,*[1] Xianfeng Zhang,†[1] Nijuan Xiang,* Yang Huai,*‡ Lei Zhou,* Zhongjie Li,* Cuiling Xu,§ Huiming Luo,¶ Jianfeng He,¶ Xuhua Guan,† Zhengan Yuan,# Yanting Li,# Longshan Xu, Rongtao Hong,** Xuecheng Liu,†† Xingyu Zhou,†† Wenwu Yin,* Shunxiang Zhang,‡‡ Yuelong Shu,* Maowu Wang,* Yu Wang,* Chin-Kei Lee,§§ Timothy M. Uyeki,¶¶ and Weizhong Yang,* for the Avian Influenza H5N1 Study Group[2]**

We investigated potential sources of infection for 6 confirmed influenza A (H5N1) patients who resided in urban areas of People's Republic of China. None had known exposure to sick poultry or poultry that died from illness, but all had visited wet poultry markets before illness.

Although >280 confirmed human cases of avian influenza A (H5N1) virus infection from 12 countries have been reported (*1*), detailed data on sources of infection for most patients are limited (*2*). In Vietnam, 8 of 9 patients with influenza A (H5N1) reported close contact with sick or dead poultry (*3*). In Thailand, 9 of 12 such patients lived in households where backyard chickens died, and 8 reported direct contact with dead chickens (*4*). Case-control studies in Thailand and Vietnam found that the most statistically significant risk factor was recent exposure to sick or dead poultry, especially directly touching dead poultry (*5*,*6*).

*Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China; †Hubei Center for Disease Control and Prevention, Wuhan, People's Republic of China; ‡International Field Epidemiology Training Program, Bangkok, Thailand; §National Institute for Viral Disease Control and Prevention, Beijing, People's Republic of China; ¶Guangdong Center for Disease Control and Prevention, Guangzhou, People's Republic of China; #Shanghai Center for Disease Control and Prevention, Shanghai, People's Republic of China; **Fujian Center for Disease Control and Prevention, Fuzhou, People's Republic of China; ††Sichuan Center for Disease Control and Prevention, Chengdu, People's Republic of China; ‡‡Shenzhen Center for Disease Control and Prevention, Shenzhen, People's Republic of China; §§World Health Organization, Beijing, People's Republic of China; and ¶¶Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Avian influenza (H5N1) poultry outbreaks have been reported in mainland People's Republic of China since 2004 (*7*); since late 2005, human cases have also been reported (*8*). Most Chinese patients had exposure to backyard poultry, although some had no apparent direct exposure to poultry that were sick or died. We describe findings of investigations of urban patients with influenza A (H5N1), who had no known direct contact with sick poultry or poultry that died of illness in China.

The Study

Enhanced surveillance for influenza-like illness and pneumonia of unknown origin was established in China after the outbreak of severe acute respiratory syndrome (SARS) (*9*). All suspected cases of influenza A (H5N1) are reported through a national surveillance system to the Chinese Center for Disease Control and Prevention (China CDC). Laboratory testing is performed by the National Influenza Center of China CDC. A confirmed case of influenza (H5N1) was defined according to World Health Organization case definitions (*10*). This study was part of an ongoing public health outbreak investigation and determined by the ministry of health to be exempt from institutional review board assessment.

China CDC conducted epidemiologic investigations by interviewing confirmed influenza (H5N1) patients and their relatives, reviewing medical records, and visiting patient households and places visited by patients within 2 weeks of illness onset. A standardized questionnaire was used to collect demographic, clinical, and exposure history data. For 3 severely ill patients, only relatives and contacts were interviewed to assess possible influenza (H5N1) subtype exposures. Particular attention was paid to potential exposures such as contact with well-appearing, sick, or dead poultry; visits to poultry markets; or contact with persons with febrile respiratory symptoms in the 2 weeks before onset. A rural case was 1 that occurred in a village resident; an urban case was 1 that occurred in a city resident.

From October 2005 through October 2006, 20 confirmed cases were reported from 12 provinces. Six cases were identified in 6 cities of 5 provinces; each city had an average population of 8.3 million and was at least 112 km away from cities with another case (Figure 1). More urban cases were reported in 2006 (5 [42%] of 12) than in 2005 (1 [13%] of 8), but this difference was not statistically signifi-

[1]These authors contributed equally to this work.

[2]Members of the Chinese Center for Disease Control and Prevention Avian Influenza H5N1 Study Group are Shuyun Xie, Zhitao Liu, Xuefeng Tang, and Jun Li. Members of the Provincial Center for Disease Control and Prevention Avian Influenza H5N1 Study Group are Gongping Liu, Jianming Ou, Shaojian Cai, Ting Huang, Tie Song, De Wu, and Xiaodong Sun.

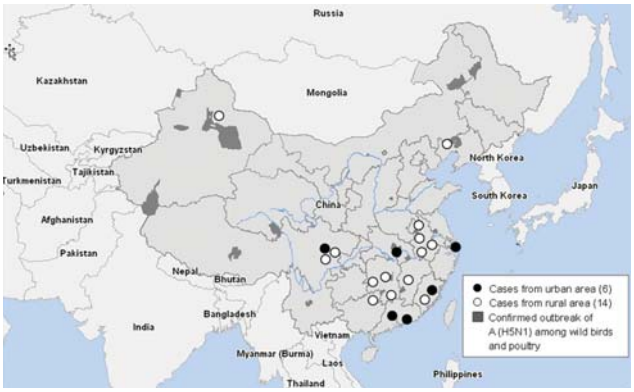


Figure 1. Map showing distribution of 6 human influenza (H5N1) cases from urban areas of People's Republic of China, compared with 14 cases from rural areas. The 6 urban cases were distributed sporadically in 6 large cities of 5 provinces, and none was associated with confirmed H5N1 subtype poultry outbreaks or sick and dead poultry.

cant (p = 0.325) (Figure 2). Demographic and clinical characteristics of the 6 urban patients with influenza (H5N1) cases are presented in Table 1. All 6 urban patients were adults, median age 30 years (range 21 to 41); 5 died.

Five of the 6 urban case-patients had no direct contact with poultry. One patient prepared freshly slaughtered chicken that she purchased for cooking at a live (wet) poultry market. No patients kept poultry or other animals at home, and no poultry or poultry outbreaks were identified in their neighborhoods. Five patients had visited wet poultry markets within a week of illness onset, and all had visited a wet market during the 2 weeks before their illness. Three patients visited wet markets at least once a day before illness onset. Only 1 patient (case-patient 5) had any travel history in the 2 weeks before illness onset. That patient had visited his parents' home in a rural area, where healthy backyard poultry were kept outside the house, and he had visited a wet market in the same area 2 weeks before illness onset (Table 2).

All 6 patients had no known contact with other confirmed influenza A (H5N1) patients or with anyone with febrile respiratory symptoms. A total of 640 persons were followed up for medical observation for 2 weeks, including 136 close contacts of the 6 patients, 389 healthcare workers who provided care for them, and 115 persons who worked in the poultry markets visited by the patients. Febrile respiratory illness developed in 5 contacts: case-patient 1's mother, case-patient 3's girlfriend, case-patient 6's daughter, a nurse who cared for case-patient 6, and a patient hospitalized on the same ward as case-patient 6. All 5 ill contacts recovered, and all respiratory specimens collected from them tested negative for influenza A (H5N1) by reverse-transcriptase–PCR. Paired acute- and convalescent-phase serum samples collected from these 5 ill contacts tested negative for subtype (H5N1) neutralizing antibodies by microneutralization assay.

Conclusions

Our study suggests that exposure to wet poultry markets may be an important influenza A (H5N1) risk factor for persons in urban areas of China. None of the 6 case-patients had known direct contact with poultry that were sick or died of illness. Two patients (case-patients 1 and 3) had no identified potential exposures except for visiting a wet poultry market during the week before illness onset. Four other case-patients visited wet markets, although other exposures could have potentially led to virus transmission. Case-patient 2 was an egg seller and could have also been infected by contact with fecally contaminated eggs. In 2005, influenza A (H5N1) virus was isolated from eggs brought to China by travelers from Vietnam (*11*). Case-patient 4 could potentially have been exposed to the virus through preparation of freshly slaughtered chickens purchased at a wet market. Case-patient 5 could have been exposed to the virus by visiting his parents' home, which had healthy backyard poultry outside, or by transporting eggs. Case-patient 6 could have been exposed to the virus at home when his wife prepared a freshly slaughtered chicken purchased from a wet market. No epidemiologic evidence suggested human-to-human transmission of influenza A (H5N1) associated with the urban patients.

These observations are consistent with results of a case-control study conducted during the 1997 influenza A (H5N1) outbreak in Hong Kong Special Administrative Region, which found the most statistically significant influenza (H5N1) risk factor was visiting a live poultry market the week before illness onset (*12*). During that outbreak, widespread subtype (H5N1) poultry dieoffs occurred in wet markets, but these dieoffs have not been observed in urban China. The role of poultry vaccination in decreasing poultry outbreaks in wet markets in China is unknown. A recent simulation study showed that silent spread of the virus can occur in poultry because of incomplete protection

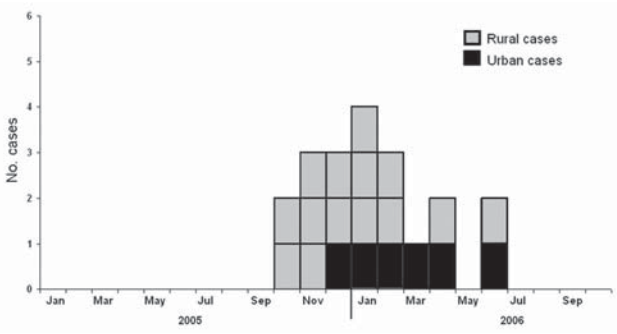


Figure 2. Number of human influenza (H5N1) cases in urban and rural areas, by month of onset, People's Republic of China, October 2005–September 2006.

Table 1. Characteristics of 6 human influenza A (H5N1) case-patients from urban areas of China

Characteristic	Case-patient no.					
	1	2	3	4	5	6
Location (city, province)	Sanming, Fujian	Chengdu, Sichuan	Guangzhou, Guangdong	Shanghai, Shanghai	Wuhan, Hubei	Shenzhen, Guangdong
Residence	Apartment	Apartment	Apartment	Temporary, at construction site	Dormitory	Apartment
Illness onset date	Dec 6, 2005	Jan 10, 2006	Feb 22, 2006	Mar 13, 2006	Apr 1, 2006	Jun 3, 2006
Days from onset to investigation	6	7	6	8	10	8
Contact with ill persons before onset	No	No	No	No	No	No
Days from onset to first medical visit	2	4	3	2	5	4
Days from onset to hospital admission	3	7	5	9	11	7
Primary signs and symptoms at admission	Fever, headache, cough	Fever, cough, diarrhea, myalgia	Fever, chills, cough, shortness of breath	Fever, chills, cough	Fever, headache, sore throat, cough, myalgia	Fever, chills, productive cough, back pain
Complications*	Respiratory failure, ARDS, cardiac failure, septicemia	Respiratory failure, ARDS, cardiac failure, septicemia	Respiratory failure, ARDS	Respiratory failure, ARDS, cardiac failure	Respiratory failure, ARDS, cardiac failure, septicemia	Respiratory failure, ARDS
Outcome	Died	Died	Died	Died	Died	Survived
Days from onset to death	16	14	9	9	19	Discharged 61 d from onset

*Respiratory failure was defined as the need for ventilator support; ARDS, acute respiratory distress syndrome, is a life-threatening condition in which inflammation of the lungs and accumulation of fluid in the air sacs (alveoli) lead to low blood oxygen levels; cardiac failure was defined as the requirement of isotropic agents.

at the flock level, even if a poultry vaccine is effective in individual birds (*13*).

In China, wet markets are sustained by demand for freshly slaughtered poultry. Wet markets are considered a reservoir and amplifier of avian influenza A viruses because they bring together avian host species in a high-density setting that can facilitate viral persistence, cross-species infection, and genetic reassortment (*14*). Our findings suggest that wet markets pose a risk that is likely to be low for avian-to-human transmission of subtype (H5N1) in ur-

Table 2. Exposure history of 6 influenza A (H5N1) case-patients from urban areas of China

Case-patient no.	Epidemiologic information
1	41-y-old female factory worker, previously healthy, with a history of thymectomy for benign thymoma 6 wk before illness onset, had recovered fully, did not require any medications, and resumed working. She visited a wet market nearly every day the week before illness onset but did not purchase poultry or poultry products. No poultry were kept in her home or neighborhood.
2	29-y-old woman, previously healthy, worked at a stall that she owned at a wet market, selling groceries and eggs. Her stall was ≈20 m away from stalls selling and slaughtering live poultry. No poultry were kept in her home or neighborhood.
3	32-y-old man, previously healthy, quit his job 1 mo before illness onset and was planning to start his own food business. He visited up to 9 wet markets for 10–40 min every day during the week before illness onset. At 1 wet market, he spent most of his time in a sauce store that was ≈5 m away from stalls where poultry were slaughtered and sold. No poultry were kept in his home or neighborhood.
4	29-y-old woman, previously healthy, moved from Guangdong to Shanghai 2 mo before illness onset. She worked as a cook for 14 people at a construction site, where she lived temporarily, and visited a wet market every day to buy fresh food, including freshly slaughtered chickens, 1 wk before illness onset. No poultry were kept in her home or neighborhood.
5	21-y-old man, previously healthy, was a security guard for an aircraft-repairing factory in Wuhan. Two wk before illness onset, he traveled to his hometown in the rural area of Enshi to attend the funeral of his uncle, who died of esophageal cancer. The man stayed there for 6 d, visited his parents' home, where healthy backyard poultry were kept (none became sick or died), and visited a wet market. One wk before onset, he traveled back to his workplace in Wuhan, bringing 200 eggs from his hometown. In Wuhan, he had no direct contact with poultry, and he did not visit any wet markets. No poultry were kept in his home or neighborhood.
6	31-y-old man, previously healthy, worked as a truck driver for shoe factories in Shenzhen city. Two d before illness onset, he visited a wet market once, but he did not purchase any poultry or poultry products. One wk before onset, his wife visited the same market and brought a live chicken that was slaughtered at the market. No abnormal dieoffs of poultry were reported. No poultry were kept in his home or neighborhood.

ban settings. Viral RNA for subtype (H5N1) was detected in a specimen collected from a goose cage at an urban wet market visited by case-patient 3 (*15*), which suggests the potential for influenza (H5N1) transmission through environmental contamination.

We were limited by inability to elicit complete exposure histories from all case-patients because of their severe illness or death. However, we interviewed household and family members, friends, and co-workers, and investigated places that patients had visited in the 2 weeks before illness onset. We were unable to ascertain how human infection with the virus occurred through the patients' visits to wet poultry markets. Possibilities include self-inoculation of the respiratory tract after touching subtype (H5N1)–contaminated surfaces, or inhalation of aerosolized debris with influenza (H5N1) virus.

We did not perform testing on poultry or environmental specimens and can only speculate about potential exposures and sources of influenza A subtype (H5N1) infection in wet markets, homes, and neighborhoods. Future studies should test tracheal, cloacal, and blood specimens from poultry; swabs of fecal material, cages, and other potentially contaminated surfaces; and air specimens in wet markets, for evidence of influenza (H5N1). More research studies, including case-control studies, are needed to better clarify the risk for subtype (H5N1) transmission that occurs from visiting wet poultry markets. Close collaborations are needed between animal health and public health agencies to reduce the public health risk for this virus in wet poultry markets and to understand the impact of poultry vaccination on the risk for transmission.

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Dr Yu is a medical epidemiologist in the Office for Disease Control and Emergency Response, China CDC. His research interests include surveillance, prevention, and control of emerging infections, including SARS, avian influenza, and *Streptococcus suis*.

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Address for correspondence: Weizhong Yang, Chinese Center for Disease Control and Prevention, 27 Nanwei Rd, Beijing, 100050, People's Republic of China; email: yangwz@chinacdc.cn

Live Poultry Exposures, Hong Kong and Hanoi, 2006

Richard Fielding,* Tran H. Bich,† La Ngoc Quang,† Wendy W.T. Lam,‡ Gabriel M. Leung,* Truong Q. Tien,† Ella Y.Y. Ho,* and Le V. Anh†

Since 1997, the largest epidemic of highly pathogenic avian influenza (H5N1) ever recorded has caused 172 human and several billion bird deaths. Recently administered questionnaires determined that live poultry exposures have declined by ≈63% in Hong Kong since 2004 and that, in Vietnam, domestic backyard exposures to poultry are likely more important than retail exposures.

Most human cases of highly pathogenic avian influenza H5N1 (HPAI) arise from exposure to infected poultry (*1*–*3*; Figure). Mapping poultry exposure and its determinants can enhance HPAI surveillance (*4*). We compared live poultry exposures in both Hong Kong Special Administrative Region and Vietnam in 2006 and examined changes in levels of exposure in Hong Kong since 2004, when a similar survey was performed in Hong Kong (*4*).

The Study

In Hong Kong, random household telephone interviewing of 1 adult >17 years of age selected by Kisch grid (which randomizes selection of persons within households) was conducted from December 2005 through 2006 from a list of 5,000 numbers. Simultaneously in Vietnam, stratified cluster sampling was carried out throughout 2 districts in each of 5 northern provinces. Within 3 of these provinces, 1 district with and 1 without an HPAI epidemic history were selected. Within each district, 1 urban and 1 rural commune each provided 100 households randomly selected from electoral rolls. Kisch grid selected 1 adult from each household for face-to-face interviews.

Respondents estimated their live poultry purchase frequency and touching at purchase (*4*). We attributed standard values to respondents' reports (e.g., monthly = 12; weekly = 52) (*4*) to give standardized household purchases of live poultry. Multiplying standard purchases by reported buying frequencies standardized buying patterns (*4*). Self-reported buyer touching of birds during purchase was standardized by adjustment for reporting differences by gender proportion weighting (*4*) and reported touching frequency to calculate adjusted buying exposures. Vietnam also surveyed backyard poultry practices. Households raising poultry reported the number, type, changes in husbandry practices, and poultry deaths for the past 12 months.

Conclusions

In Hong Kong, 2,784 contacts yielded 1,760 interviews (return rate 63%); 64% of respondents were women and 36% were men; their median age, 44 years (Table 1). Vietnam's census-derived sample frame comprised 2,412,000 of 18,264,000 national households; 1,988 (0.01% of all Vietnamese households) formed the sample. Of 1,196 (60%) female and 792 (40%) male participants, the median age was 39 years, 50% lived in urban and 50% in rural communes, and >11% had primary education only (Table 1).

In Hong Kong in 2006, 18,586 standardized purchases averaged 10.56 chickens/household/year (for men, 9.4, for women, 11.2). This is a territory-wide gender-adjusted rate of 11.05 chickens/household/year, which indicated that 22,673,000 live chickens were purchased during the preceding year, 41% fewer than in 2004. Households buying poultry bought an average of 15.6 chickens/household/year. Among respondents personally buying, 7.5% touched the poultry during purchasing (compared with 11% in 2004), giving ≈1,700,500 exposures/year. Adjustment for touching frequency and gender differences in reported touching (males 6.7%, females 4.7%) gave ≈1,110,900 contacts (4.9%, 95% confidence limit [CL] 3.8–6) for Hong Kong in 2006, or 0.76 exposures/buying household/year (0.23/person/year, if one assumes 3.36 persons/household). Applied retrospectively to Hong Kong 2004 data, this gave an adjusted exposure rate of 8.6% (95% CL 6.8–10.3), ≈3,311,300 contacts, and exposure frequency of 2.07 ex-

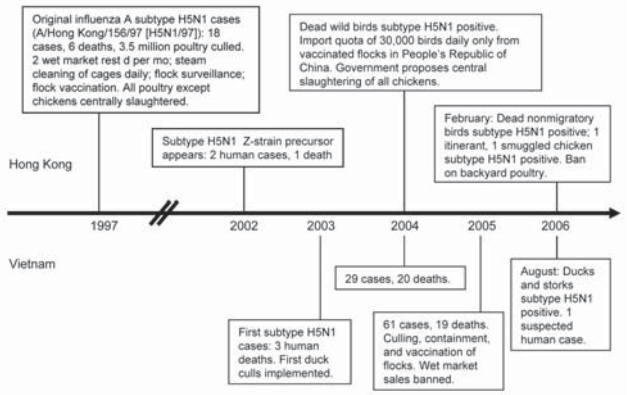

Figure. Chronology of influenza A (H5N1) outbreaks and responses, Hong Kong and Vietnam. Double slashes represent a break in the timeline.

*University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China; †Hanoi School of Public Health, Ha Noi, Vietnam; and ‡Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region, People's Republic of China

Table 1. Sample characteristics and population censuses, Hong Kong and Vietnamese samples

	Survey Vietnam; Hong Kong, %	Census Vietnam*; Hong Kong†, %	Effect size‡ Vietnam; Hong Kong
Sex			
Male	39.8; 35.9	46.1; 47.8	0.19; 0.24
Female	60.2; 64.1	50.9; 52.2	
Age, y			
15–24	14.2; 10.5	28.1; 11.1	0.50; 0.21
25–34	22.5; 14.4	21.7; 18.1	
35–44	25.3; 27.0	20.4; 22.7	
45–54	22.4; 27.0	13.7; 21.2	
55–64	13.2; 10	6.9; 11.9	
≥65	2; 11	9.3; 14.9	
Residence			0.54
Urban	50	26.3	
Rural	50	73.7	
Education			0.32
None or kindergarten	3.9	8.4	
Primary	14.7	20.5	
Secondary	49.5	45.2	
Matriculation	6.3	9.4	
Tertiary/above	25.3	16.4	

*Reference (*5*).
†Reference on education (*6*). On sex and age, mid-2006, available from www.censtatd.gov.hk/hong_kong_statistics/statistical_tables/index.jsp
‡Three levels of effect size: 0.1 small, 0.3 medium, 0.5 large.

posures/buying household/year (0.62/person/year) in 2004. These adjusted estimates indicate an absolute exposure decline of 3.7% (95% CL 2.25%–4.91%), a relative decline of 43% between 2004 and 2006. Less purchasing and touching reduced annualized buying exposures by 63% overall.

In Vietnam, respondents reported 10,659 standardized purchases, averaging 5.36 chickens/household/year, giving a gender-adjusted (male 5.5, female 5.3) rate of 5.43 chickens/household/year. Estimated number of live birds purchased in the sampled provinces (5.43 × 2,412,000 households) was 13,097,000 chickens per year. Buying households (820,080, 34%) buy on average 15.97 chickens per year, comparable to the Hong Kong 2006 purchase rate. Touching frequency during purchasing (overall 68%, 64%–71%; women 70% [67%–73%], men 54% [51%–57%]; $\chi^2 = 45.57$, df = 4, p<0.001), after adjustment for gender proportion and reported touching, was 63% (62%–64%). Estimated exposures in the surveyed provinces from buying were ≈13,097,000 × 0.63 = ≈8,251,000 exposures/year. When these rates were used, national per capita exposure estimates (assuming 4.49 persons/household) from touching when buying are ≈62,479,000 exposures/year, 2.24 exposures/person/year in buying households, 0.76 exposures/person/year overall.

In the 1,150/1,988 households (58%) that raised poultry, 92 (5%) ceased keeping poultry from February 2005 through February 2006 (Table 2). Households kept a median of 9 chickens. Overall, 22% of those keeping backyard poultry reportedly had birds die in the previous year. Of these, 12% of households threw the dead bird away without informing authorities, 9% informed the authorities, and 5% sold or ate the dead bird. Of those reporting bird deaths, 214 (84%) had been ordered by officials to destroy some or all of their birds. Incidence of bird deaths was greater in rural areas (52% vs. 48%, Fisher p<0.001), but rural residents threw them away (68% vs. 32%, Fisher p = 0.031) or sold or ate them (87% vs. 13%, Fisher p = 0.006) more often than did urban residents.

While 34% (32%–36%) of households buy live chickens, 53% (52%–54%) (1,278,360) raise live poultry at home, and 12% (10%–13%) do both. Assume a 53% national average and, conservatively, that all persons within households rearing backyard poultry have at least weekly physical contact with their birds, bird eggs, or feces. House-

Table 2. Numbers of households rearing domestic poultry, Vietnam, 2006 (proportions)

Poultry	No. households			
No. birds raised	1–5	6–10	11–20	≥21 (range)
Type				
Fighting cocks	40	5	1	2 (21–50)
Chickens	284	283	257	155 (21–800)
Ducks	52	14	5	14 (21–500)
Geese/swans	42	22	11	8 (21–70)
Ornamental	32	10	4	1 (21–30)

hold size in the surveyed districts averages 3.38 persons (General Statistics Office, Hanoi). Thus, 224,685,500 exposures/year would occur from backyard poultry in surveyed districts, an average exposure within backyard poultry raising households of ≈175 exposures/person/year. Households buying live poultry have 8,251,000 /820,080 = 10.1 exposures/household/year (2.99 exposures/person/year) from these purchases. Total purchase-related plus backyard exposure events then equal (10.1 × 820,080) + 224,685,500 = 232,968,300 exposures/year. Average household exposure is therefore ≈96 exposures/household/year (28 exposures/person/year) in sampled districts. If daily backyard exposure occurs, then there are ≈1,581,081,300 total exposures, ≈655 exposures/household/year (194 exposures/person/year). Nationally, average household size is 4.49 persons. Hence, between ≈2,322,546,000 (weekly contact) and ≈15,882,953,000 (daily contact) exposures/year, ≈127–869 exposures/household/year occur nationally. If multiple contacts occur daily, these figures would be much higher.

Epidemic and nonepidemic district-buying frequency CL overlapped (exposure 3.4 [1.9–4.8] chickens/household/year vs. nonexposure 5.8 [4.5–7.0] chickens/household/year). Dual adjusted touching frequencies were 69% (62%–76%) in epidemic and 60% (57%–63%) in nonepidemic districts, respectively. Backyard poultry were more common in epidemic districts (71% [67%–75%] vs. 45% [42%–48%]), where keeping poultry declined 17% (14%–20%) compared with 8% (6%–10%) in nonepidemic districts. Epidemic and nonepidemic districts had comparable average incomes ($t = 0.832$, df 1,283.9, $p = 0.406$).

In Hong Kong, government import restrictions have reduced poultry availability by 41% from 2004 to 2006. Purchase and touching declines prompted by health education messages have together reduced exposure by ≈60%.

Fewer Vietnamese households bought live chickens, but those that did so bought at comparable frequencies to Hong Kong 2006 households. Chickens are relatively more expensive in Vietnam. Adjusted for purchasing power parity (www.worldbank.org/data/quickreference/quickref.html), live chickens costs $16.6–$18.0 and $21.8–$31.0 (international dollars; www.worldbank.org/data/quickreference/quickref.html) each in Hong Kong and in Vietnam, respectively. Hence, temptation to use sick, dying, or dead poultry is high, increasing the risk for human influenza (H5N1) infection (*7*). Average Vietnamese exposure range from backyard sources (28–194 exposures/person/year) is 100–700× higher than Hong Kong 2006 exposures from purchasing (0.23 exposures/household/year). If 53% of Vietnamese households average 9 birds each and if 22% of these households (2,129,582) had only 1 bird die, a 5% consumption rate of the dead birds means that 106,500 sick or dying birds are consumed annually, posing a major health threat (*7*). This is a risk that governments must urgently target.

Limitations include generalizing from 5 northern Vietnamese provinces to the country as a whole and using arbitrary estimates for backyard exposure frequency. Nonetheless, valuable data are presented on differential exposure patterns.

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Dr Fielding is professor of medical psychology and head of the Behavioral Sciences Unit at the Department of Community Medicine and School of Public Health, University of Hong Kong, People's Republic of China.

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Address for correspondence: Richard Fielding, Department of Community Medicine and Unit for Behavioural Sciences, 5/F, WMW Mong Bldg, The University of Hong Kong, 21 Sassoon Rd., Pokfulam, Hong Kong Special Administrative Region, People's Republic of China KHSAR; email: fielding@hkusua.hku.hk

Cutaneous Leishmaniasis, Sri Lanka

Sujeevi S.K. Nawaratna,* Danister J. Weilgama,* Chandana J. Wijekoon,† Manel Dissanayake,‡ and Kosala Rajapaksha§

Cutaneous leishmaniasis (CL) is an emerging disease in Sri Lanka. Of 116 patients with clinical symptoms suggestive of CL, 86 were confirmed positive for *Leishmania donovani*. Most patients had single dry lesions, usually on the face. Patients were from 5 of the 7 agroclimatic zones in Sri Lanka.

Leishmaniasis is a complex of diseases that has 3 main clinical forms, visceral, mucocutaneous, and cutaneous. For decades in Sri Lanka, leishmaniasis was considered an exotic disease. This changed in 1992 with the detection of locally acquired cases of cutaneous leishmaniasis (CL) (*1*,*2*). In 2003, Karunaweera et al. (*3*) reported that CL in Sri Lanka is caused by the parasite *Leishmania donovani* zymodeme MON-37; however, information on other aspects of the disease in Sri Lanka was scant. We present preliminary findings on the clinical manifestations and the distribution of CL in Sri Lanka.

The Study

The study group consisted of patients referred by their dermatologists from June 2001 through June 2005 for skin lesions clinically suggestive of CL. The patients were examined for confirmatory diagnosis. Ethical clearance for the study was granted by the Research and Higher Degrees Committee of the Faculty of Medicine, University of Peradeniya, Sri Lanka.

Laboratory diagnosis was made by examination of Giemsa-stained touch or impression smears, in vitro culture, and/or by PCR. Tissue samples for cultures and molecular testing were obtained by use of a hypodermic needle. Evans modified Tobie medium was used for in vitro culture (*4*). PCR for diagnosis was performed on DNA extracted from tissue samples, by using a set of primers specific for all Old World *Leishmania* spp. (*5*).

Leishmania DNA from 27 patients positive for CL and from 5 in vitro cultures (promastigotes) was sent to Laboratoire de Parasitologie, Besançon, France, for speciation. Characterization was performed by use of microsatellite analysis (also known as short tandem repeats), which used primers to amplify microsatellites in the internal transcribed spacer region of the *Leishmania* genomic DNA (*6*).

Of 120 patients examined, 4 had a history of travel overseas and were not included in the analysis. Of the remaining 116 patients, 86 (74.14%) were determined to be positive for CL. All but 2 of the 32 samples that were sent for speciation were identified as *L. donovani*; the 2 that were not identified did not have sufficient DNA for testing.

At the time of study, patients had CL-related lesions ranging in duration from 2 weeks to 4 years. Both male and female patients were infected (ages range 3–70 years).

All infected patients had cutaneous lesions only; none showed hepatosplenomegaly or enlargement of lymph nodes. Single lesions were seen in 70 of the 86 patients, and multiple lesions (range 2–5) were observed in the other 16 patients. Satellite lesions were seen in 11 of the 86 patients. Lesions appeared on the face of most patients (54.7%), but they also occurred in ears and on upper and lower limbs and the trunk (Table 1). No lesions were found from the waist to the knee.

The lesions on most patients were dry and scaly (69/86), but on some, they were wet (17/86). A few patients showed a hypopigmented halo around the lesions. Patients' lesions were categorized according to their appearance: of the 86 patients, 25 had papulonodular lesions, 25 had noduloulcerative lesions, and 36 had ulcerative lesions. Some ulcers had the typical volcanic appearance.

The CL-infected patients came from 12 administrative districts (Figure), representing 8 of the country's 9 provinces. The areas in which the patients lived were representative of 5 of the 7 agroclimatic zones within Sri Lanka (Table 2).

Conclusions

This study supports the earlier identification (*3*) of *L. donovani* as the causative agent of CL in Sri Lanka. The

Table 1. Distribution of cutaneous leishmaniasis lesions on infected patients, Sri Lanka, June 2001–June 2005

Location	No. patients*	%
Face	47	54.7
Scalp	1	1.2
Ear	5	5.8
Neck	3	3.5
Trunk	7	8.1
Upper limb		
Upper arm	4	4.7
Elbow	1	1.2
Forearm	22	25.6
Hand	2	2.3
Lower limb		
Above knee	0	0
Below knee	7	8.1
Foot	1	1.2

*Some had multiple lesions at different sites.

*University of Peradeniya, Peradeniya, Sri Lanka; †Teaching Hospital, Kurunegala, Sri Lanka; ‡Teaching Hospital, Kandy, Sri Lanka; and §Veterinary Research Institute, Peradeniya, Sri Lanka

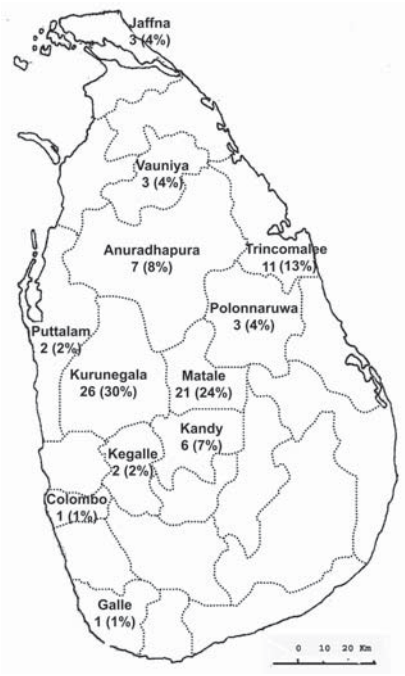

Figure. Geographic distribution of persons with cutaneous leishmaniasis in Sri Lanka during June 2001 through June 2005.

27 isolates used in our study were from patients from 7 districts (Kandy, Matale, Kurunegala, Anuradhapura, Vauniya, Trincomalee, and Jaffna) in 5 Sri Lankan provinces (Central, North Central, North Western, Northern, and Eastern Provinces), representing different agroclimatic zones of the country. Thus, *L. donovani* appears to be the only species causing CL in Sri Lanka.

L. donovani usually causes visceral leishmaniasis, but there are other reports of it being associated with CL (*7*,*8*). A single parasite strain can cause different clinical symptoms (e.g., strains normally causing dermatotrophic symptoms instead causing visceral symptoms and vice versa) (*9*,*10*).

In our study, 68% of the infected persons were 11–40 years of age and frequently engaged in outdoor activities; similar findings have been reported from Guatemala (*11*). In addition, for patients in our study, lesions most often appeared on the face. Reports of CL from other countries and caused by different species have indicated similar findings (*8*,*12*). Lesions also frequently occurred on the forearms of our study population, which suggests that uncovered areas of the body are more prone to clinical manifestation of CL infection. Sharma et al. (*8*) also reported that lesions most commonly appeared on the face and then the upper limbs; an earlier report from Sri Lanka showed extensor surfaces of the limbs to be the most common site for lesions (*2*). We also found skin lesions on the chest and back of some adult males; however, lesions did not occur in these sites on females. This finding is a direct reflection of the cultural habits related to clothing in Sri Lankan society. Also of interest, none of the patients had lesions in the area from below the waist to above the knees. This finding again is due to clothing habits. Dedet et al. (*13*), in their study on CL in French Guiana, found the distribution of lesions on the body to depend on the form of dress. One patient in our study group had a lesion on the scalp. This appears to be an unusual site, as hairy parts of the body were otherwise unaffected.

The lesions in our patients were slow-progressing, and 58% appeared as papulonodular and noduloulcerative type lesions. A similar clinical picture was reported by Sharma et al. (*8*) in a study in which the researchers encountered noduloulcerative plaques with or without crusting. The morphologic appearance of lesions is known to vary de-

Table 2. Distribution of persons infected with cutaneous leishmaniasis in Sri Lanka, by agroclimatic zone, June 2001–June 2005

Agricultural zone (elevation), climatic zone (rainfall/year)	Patient's district*	No. patients (N = 86)
Up country (>900 m)		
Wet (1,400 to >3,175 mm)	—	0
Intermediate (1,150 to >2,160 mm)	—	0
Mid country (300–900 m)		
Wet (1,270 to >3,175 mm)	Kandy	4
Intermediate (900 to >1400 mm)	Kandy	2
Low country (<300 m)		
Wet (1,525 to >2,540 mm)	Kegalle	2
	Colombo	1
	Galle	1
Intermediate (900 to >1,150 mm)	Kurunegala	26
Dry (<900 mm)	Puttalam	2
	Jaffna	3
	Anuradhapura	7
	Polonnaruwa	3
	Vauniya	3
	Trincomalee	11
	Matale	7

*The districts of Kandy and Matale fall into 2 agroclimatic zones.

pending on the species or strain of the causative organism (*13*) and the immune status of the patient (*14*).

Cutaneous nodules surrounded by areas of depigmentation have been described in atypical CL due to *L. chagasi* (*15*). This feature was also found in 12 of our 86 patients. Thus, the clinical manifestations of CL vary; there is not a characteristic clinical picture for disease caused by a particular species.

Most of the CL patients in our study were from the dry and intermediate zones of the low-altitude areas of Sri Lanka. No cases of CL were diagnosed in persons from high-altitude areas. This could be due to the abundance of sandflies (insect vectors for *Leishmania*) and their breeding sites in the low-altitude areas.

Since the first detection of CL in Sri Lanka in 1992, the number of cases detected annually has increased (*2*,*3*). A substantial number of persons in our study had a diagnosis of CL, but the prevalence of infection in Sri Lanka could not be determined because the study did not involve active case detection. To understand the atypical behavior of *L. donovani* in Sri Lanka, studies need to be directed toward understanding the vector bionomics and reservoir hosts for this parasite.

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Dr Nawaratna is a lecturer in the Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka. Her research interests are CL and infections due to free-living amebae.

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Address for correspondence: Sujeevi S.K. Nawaratna, Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka; email: knava@pdn.ac.lk

Norovirus in Captive Lion Cub (*Panthera leo*)

Vito Martella,* Marco Campolo,* Eleonora Lorusso,* Paolo Cavicchio,† Michele Camero,* Anna L. Bellacicco,* Nicola Decaro,* Gabriella Elia,* Grazia Greco,* Marialaura Corrente,* Costantina Desario,* Serenella Arista,‡ Krisztián Banyai,§ Marion Koopmans,¶ and Canio Buonavoglia*

African lions (*Panthera leo*) are susceptible to viral diseases of domestic carnivores, including feline calicivirus infection. We report the identification of a novel enteric calicivirus, genetically related to human noroviruses of genogroup IV, in a lion cub that died of severe hemorrhagic enteritis.

Lions (*Panthera leo*) are susceptible to viral diseases of domestic carnivores, including infections with canine distemper virus, feline parvovirus, feline retroviruses, feline herpesvirus, and feline calicivirus (FCV) (*1–4*). Antibodies to FCV have been detected in captive lions (*2*), and calicivirus-like particles have been detected in oral vesicular lesions of captive immature lions (*3*). Despite the presence of FCV-specific antibodies and the observation that cub survival may be reduced during calicivirus outbreaks, clear signs of FCV-induced illness have not been described in free-ranging lions (*4*). We detected a novel calicivirus in a 4-week-old lion cub that died of severe hemorrhagic enteritis.

The Study

In autumn 2004, the Zoo of Pistoia, Italy, adopted 2 adult lions that had been born in captivity. In October 2005 and May 2006, the female gave birth to 2 cubs each delivery, which 3–4 weeks later showed signs of enteritis and died. In October 2006, she gave birth to a single cub, which died of severe hemorrhagic enteritis at 4 weeks of age. The cub exhibited anorexia, depression, and mild dehydration, but it was not moved away from the mother for ethologic and management reasons. In the subsequent days, the cub's general condition appeared to worsen; anorexia and more marked depression were reported by the animal caretakers. Therefore, on day 3 after illness onset, the cub was taken to the zoo's animal hospital. Examination showed a temperature of 38.6°C, hemorrhagic enteritis, tenesmus, and deep sensorial depression. Hydration and antimicrobial therapy were immediately started, but after 24 hours the animal was agonal and hypothermic and was therefore euthanized. At necropsy, severe hemorrhagic enteritis, hemorrhage in the intestinal lymph nodes, and marked dehydration were observed. Histologic examination showed marked alteration of the intestinal mucosa: erosions, villi depletion, and hemorrhagic infiltration.

The tissues and intestinal contents were screened for common feline and canine viral pathogens by using either conventional or quantitative PCR and reverse transcription–PCR (RT-PCR). Results were negative for known feline (parvovirus, coronaviruses, herpesvirus, retroviruses) and canine (distemper virus, parvovirus, adenoviruses type-1 and type-2) pathogens. Calicivirus was identified in the intestinal content by using a broadly reactive primer pair, p289-p290, targeted to highly conserved motives of the RdRp region of the polymerase complex (*5*), but unexpectedly, the strain could not be characterized as FCV by using multiple sets of primers specific for the FCV capsid gene. In addition, the sample was positive for the norovirus (NoV)-specific primer pair JV12Y-JV13I (*6*).

Bacteriologic investigations detected an *Escherichia coli* O86, enteropathogenic *E. coli* (EPEC) group. *Clostridium sordelli* and *C. perfringens* were also isolated. By screening of the *cpb, cpb2, etx,* and *cpe* genes, the *C. perfringens* isolate was characterized as toxin-type A.

Sequence analysis of the 315-bp fragment of the RdRp region (strain 387/06) by using BLAST (www.ncbi.nlm.nih.gov/blast) and FASTA (www.ebi.ac.uk/fasta33) showed that the virus was distantly related to FCV (<35% amino acid [aa] identity) but closely related to human and animal NoVs (≤ 75% aa identity). To determine the sequence and genome organization of the novel calicivirus, a 3.4-kb region at the 3′ end of the genome was amplified by RT-PCR as described by Wang et al. (*7*). The sequence of the 3′ end of open reading frame (ORF)1, the full-length ORF2, ORF3, and the noncoding region through the poly-A tail was determined (GenBank accession no. EF450827). A 14-nt overlap was present in the ORF1–ORF2 junction region, as it is in most human and animal NoVs. The ORF2 was 1,737 nt long and contained an ORF encoding a capsid protein with a predicted size of 578 aa. By BLAST and FASTA analysis, the highest sequence match was found to genogroup IV NoVs (69.3–70.1% aa identity), and identity to non-GIV NoVs was ≤52.6% aa. A total of 23 aa insertions, scattered throughout the P2 domain, were present in the capsid protein of the lion NoV when compared with human genogroup IV NoVs. A 1-nt overlap was found between ORF2 and ORF3, and a 106-nt long nontranslated

*University of Bari, Valenzano, Bari, Italy; †Giardino Zoologico di Pistoia, Pistoia, Italy; ‡University of Palermo, Palermo, Italy; §Baranya County Institute of State Public Health Service, Pécs, Hungary; and ¶National Institute of Public Health and the Environment, Bilthoven, the Netherlands

region was found between ORF3 and the poly-A tail. ORF3 was 765 nt long and encoded for a 254-aa polypeptide. The nucleotide identity plot of the genome of the lion NoV (from the 3′ end of ORF1 to the poly-A tail) was compared with the human genogroup IV.1 NoV, Fort Lauderdale/560/98/US (AF414426) (Figure 1). A phylogenic tree was constructed by using the capsid protein of a selection of human and animal NoVs of the various NoV genogroups (I to V) (*7*,*8*). In the tree (Figure 2), the lion calicivirus strain was grouped with genogroup IV human NoVs.

Conclusions

NoVs in humans were first discovered by use of electron microscopy in 1972 (*10*). As a consequence of the development and large-scale application of new and sensitive molecular diagnostic techniques, NoVs are now regarded as the major cause of epidemic, nonbacterial gastroenteritis worldwide in humans of all age groups (*9*). Human NoVs are classified into genogroups I, II, and IV. In addition, NoVs classified in genogroups II and III have been detected in pigs and cows (*7*,*11*,*12*), and NoVs proposed as genogroup V have been detected in mice (*13*) (Table). However, to our knowledge, NoVs have not been detected in other animal species and our report is the first description of NoVs in felids.

Because of the possibility of genetic recombination, a consistent and reliable classification of NoV is necessarily based on analysis of the complete capsid gene, and a comprehensive classification scheme has been established by analysis of 164 NoV strains (*8*). Strains within the same genotype (or cluster) share >85% aa identity; strains of different genotypes within the same genogroup share 55%–85% aa identity (*8*). The lion NoV 387/06 appeared to be more related genetically to human genogroup IV NoVs (69.3%–70.1% aa identity in the capsid protein). Accordingly, the virus may be considered as a distinct genotype (IV.2) within genogroup IV; human genogroup IV NoVs are genotype IV.1.

The close genetic relationship observed between the lion NoV strain and human genogroup IV NoVs reinforces the notion that the evolution of human NoVs is intermingled with that of animal NoVs. The mechanisms driving the evolution of NoVs are accumulation of punctuated mutations and recombination (*14*). In addition, NoVs can infect heterologous species, resulting in mild or unapparent infections (*15*). To assess whether animal NoVs have emerged over time in humans by direct interspecies transmission or by exchange of genetic material through recombination with human NoVs, the genetic diversity of animal NoVs must be explored.

To acquire epidemiologic information, either single or pooled fecal samples of overtly healthy animals from the zoo were screened by RT-PCR with broadly reactive or

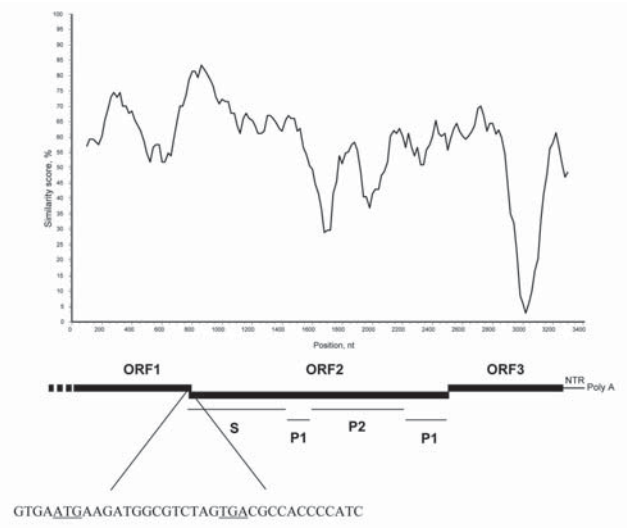

Figure 1. Genome organization of the lion norovirus (NoV) 387/06. A nucleotide identity plot of the genome of the lion NoV (from the 3′ end of open reading frame [ORF] 1 to the poly-A tail) was compared with the human genogroup IV.1 NoV, Fort Lauderdale/560/98/US (AF414426). The sequences were analyzed with Simplot software (http://sray.med.som.jhmi.edu/scroftware/simplot) by using a window size of 200 and step size of 20 with gap strip off and J-C correction on. The ORF1–ORF2 junction region is shown with the starting and stopping codons ATG and TGA underlined. The highly conserved domain S and the highly variable domains P1 and P2 of the capsid protein are also indicated.

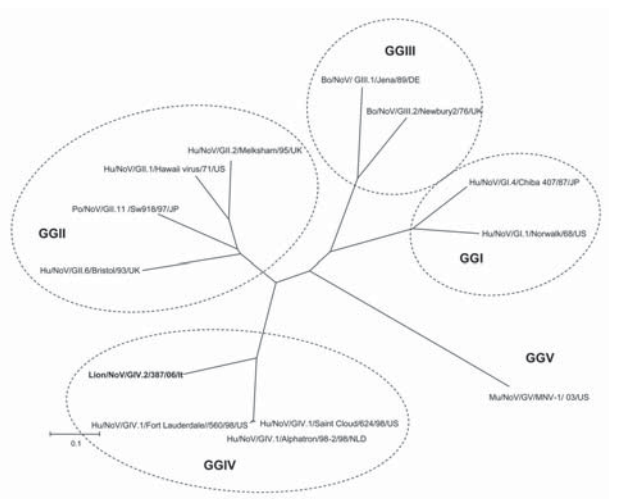

Figure 2. Phylogenetic tree constructed on the full-length amino acid (aa) sequence of the capsid protein. The tree was constructed by using a selection of norovirus (NoV) strains representative of genogroups (GG) I to V. Phylogenetic reconstruction was carried out with the p-distance correction and the neighbor-joining method, supported with bootstrapping >1,000 replications. Distance analysis and phylogenetic inference were carried out using the Mega 3.0 software package (www.megasoftware.net). Strain classification follows the outlines of Wang et al. (*7*) and Zheng et al. (*8*); strain designation follows the outlines of Green et al. (*9*). Bo, bovine; DE, Germany; UK, United Kingdom; Hu, human; JP, Japan; US, United States; Mu, murine; NLD, the Netherlands; Po, porcine.

Table. Distribution of norovirus genogroups and genotypes

Host	Genogroup and genotypes*				
	I	II	III	IV	V
Human	1–8	1–10, 12–17		1	
Pig		11, 18, 19			
Cattle			1, 2		
Lion				2†	
Mouse					1

*Norovirus classification follows the outlines of Wang et al. (*7*) and Zheng et al. (*8*).
†Determined in this study.

specific primer sets. Samples of adult and immature lions, tigers (*P. tigris*), jaguars (*P. onca*), manul cats (*Otocolobus manul)*, siberian lynxes (*Lynx lynx wrangeli*), fennecs (*Vulpes zerda*), polar bears (*Ursus maritimus*), and wolves (*Canis lupus*) were screened; calicivirus RNA was not detected.

Whether the novel lion calicivirus is a newly identified felid viral pathogen or a NoV strain of heterologous origin detected incidentally in the intestinal content of the cub remains to be proven. Bacterial coinfections were also detected and likely enhanced the severity of the enteritis disease by triggering synergistic effects. Accordingly, the pathogenic potential and the origin of the novel calicivirus strain remain to be elucidated.

Acknowledgments

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Dr Martella is associate professor at the Faculty of Veterinary Medicine of Bari, Italy. His research involves diagnosis, epidemiology, and characterization of enteric viruses, including rotaviruses and caliciviruses, in animals and humans.

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Address for correspondence: Vito Martella, Dipartimento di Sanità e Benessere Animale, Facoltà di Medicina Veterinaria di Bari, S.p. per Casamassima Km 3- 70010 Valenzano, Bari, Italy; email: v.martella@veterinaria.uniba.it

Little Evidence for Genetic Susceptibility to Influenza A (H5N1) from Family Clustering Data

Virginia E. Pitzer,* Sonja J. Olsen,† Carl T. Bergstrom,‡ Scott F. Dowell,† and Marc Lipsitch*

The apparent clustering of human cases of influenza A (H5N1) among blood relatives has been considered as evidence of genetic variation in susceptibility. We show that, by chance alone, a high proportion of clusters are expected to be limited to blood relatives when infection is a rare event.

Since December 2003, 36 family clusters among 261 confirmed human cases of influenza A (H5N1) have been documented (*1,2*). These clusters range in size from 2 to 8 infected persons; in only 4 clusters were 2 unrelated family members (e.g., husband and wife) infected. This pattern has been considered by the World Health Organization as evidence of genetic variation in susceptibility (*3–5*), but we show this observation provides little grounds for this inference. We describe a null model in which nuclear families experience a common exposure to an avian influenza virus. The observed degree of clustering in blood relatives is consistent with that expected by chance alone in the absence of genetic variation in susceptibility; other features of the data are also consistent with the null model.

Our model assumes all persons are equally susceptible, such that they have the same probability of infection, τ, and ignores possible human-to-human transmission (see online Technical Appendix, available from www.cdc.gov/EID/content/13/7/1074-Techapp.htm). The number of infected family members follows a binomial distribution with mean $n\tau$, where n is the number of exposed persons in each family. A cluster is defined as a family in which >1 person is infected; clusters are limited to blood relatives unless both parents are infected.

We compare our model to the observation that 32 of 36 clusters that occurred from December 2003 to December 2006 consisted only of blood relatives (p_B = 0.89, 95% confidence interval 0.74–0.97; Table in online Technical Appendix). When the probability of infection is low, most clusters consist of 2 infected family members, and by simple combinatorics, these 2 are usually blood relatives, which is consistent with the observed date (Figure 1).

For a given a nuclear family size, the null model also predicts the proportion of all cases that are part of a cluster and the average number of cases per cluster. Neither of these measures follows a simple distribution; we therefore use simulated data to determine what ranges of our parameters (τ and n) are consistent with the observed degree of clustering both in families and among blood relatives. We estimate the mean and 95% prediction intervals for the proportion of cases occurring in clusters when there are 261 cases, and for the average number of cases per cluster when there are 36 clusters. The expected proportion of cases occurring in clusters is similar to the observed data when the probability of infection is low (τ<0.15) (Figure 2). The observed average number of cases per cluster, however, is consistent with slightly higher probabilities of infection, larger family sizes, or both (Figure 2).

The discrepancy between the number of cases per cluster and the proportion of cases in clusters may be due to between-family variation in τ. If the probability of infection is low for members of most exposed families and higher for members of a few exposed families, then most cases may come from families in which τ is low, but most of the clusters will occur among families for which τ was higher. This will lead to a lower proportion of cases occurring in clusters and a higher average number of cases per cluster, as is observed. Although it is possible that such variation may be genetic, it could also result from between-household heterogeneity in intensity of exposure to infected birds

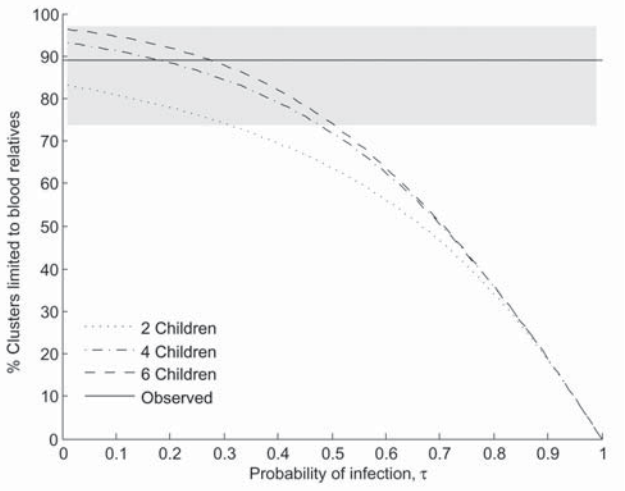

Figure 1. Proportion of clusters limited to blood relatives versus the probability of infection (τ) under the null hypothesis (no variation in susceptibility). Point estimate of the observed data is represented by the solid black line; the shaded region represents the 95% confidence interval.

*Harvard School of Public Health, Boston, Massachusetts, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ‡University of Washington, Seattle, Washington, USA

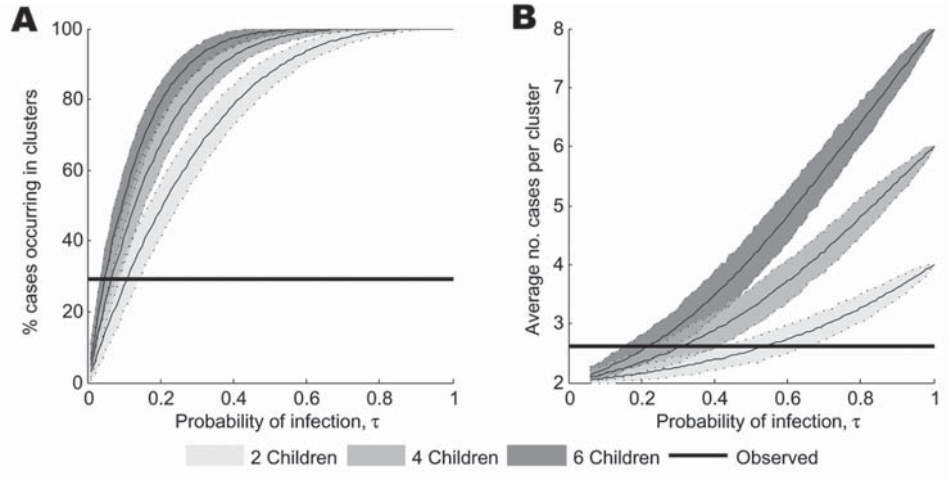

Figure 2. Relationship between data simulated under the null model and the observed pattern of family clustering for A) the proportion of cases occurring in clusters (given 261 total cases) and B) the average number of cases per cluster (given 36 clusters). Estimates of the mean are represented by solid lines; the shaded regions between the dotted lines show 95% prediction intervals for 1,000 simulations. The observed data are represented by the solid black lines.

(or intensity of shedding in birds to which different households are exposed), household hygiene, living conditions, and the like. Human-to-human transmission of the virus could also lead to larger than expected cluster sizes because having ≥1 case(s) within a family would increase the risk of subsequent cases occurring, and it could not be ruled out in several clusters (*6,7*).

Qualitatively, the data suggest the existence of nongenetic, between-household variation in risk. If such nongenetic variation were absent, then in any given village, nearly all pairs of cases occurring among unrelated persons in the same village would be in different households. Roughly, the chance that a pair of cases in unrelated persons in a village would be from the same household as opposed to different households would be $1/H$, where H is the number of households in a village. With 4 pairs of cases in unrelated persons in the same household, $\approx 4H$ pairs of cases would be expected within a village, mostly in different households. If the average village size of ≈138 households estimated for an area of Thailand (*8*) is typical, then if members of all households in a village were at equal risk, we would expect to see far more pairs of unrelated cases within a village than have actually been observed ($4H \approx 550$ pairs of cases in unrelated persons, which greatly exceeds the observed 261 total cases). Clearly, this argument is only heuristic, but when this argument is combined with the likelihood of biologic and behavioral differences between households, it seems likely that τ would vary considerably from 1 household to another.

Furthermore, the model does not account for additional individual variability in susceptibility possibly related to age, level of exposure, or other risk factors. If younger persons have a higher risk for infection or likelihood of exposure, clustering would be promoted, primarily within blood relatives, because siblings would be more likely than either parent to become infected. Approximately half of all cases have occurred in those <20 years of age (*9*). Similarly, if female persons (for example) were at higher risk for exposure, infection, or both, then clusters including non–blood relatives (e.g., spouses) would tend to include the low-risk sex and thus be less probable. Female persons of ages 10–29 years were slightly overrepresented among laboratory-confirmed case-patients, but the difference was not statistically significant (*9*).

The null model presented here is not designed to capture all of the heterogeneities in exposure and complexity of real families exposed to influenza subtype H5N1. Rather, it simply illustrates that a large proportion of family clusters limited to blood relatives may occur by chance in the absence of genetic variation in susceptibility, particularly when the probability of infection is low and family sizes are large. Although genetic heterogeneity may possibly contribute to the clustering of avian influenza cases within blood relatives, it is neither a necessary nor the most likely explanation for the data currently available.

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Ms Pitzer is a Doctor of Science candidate in the Department of Epidemiology at Harvard School of Public Health. Her research interests include epidemiologic methods for investigating emerging infectious diseases.

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Address for correspondence: Virginia E. Pitzer, Department of Epidemiology, Harvard School of Public Health, 677 Huntington Ave, Boston, MA 02115, USA; email: vpitzer@hsph.harvard.edu

Point-of-Use Water Treatment and Use among Mothers in Malawi

Lauren J. Stockman,*† Thea K. Fischer,* Michael Deming,* Bagrey Ngwira,‡ Cameron Bowie,‡ Nigel Cunliffe,§ Joseph Bresee,* and Robert E. Quick*

A national household survey was conducted in Malawi to determine awareness and use of a socially marketed water treatment product. In all, 64% of mothers were aware of the product, and 7% were using it. Both poor and rural mothers had lower awareness and use rates. Targeting promotion to rural populations could enhance program effectiveness.

Diarrhea is a leading cause of childhood deaths in the developing world (*1*), where many people rely on drinking water that is contaminated with pathogens. To address this problem, the Centers for Disease Control and Prevention (CDC) and the Pan American Health Organization/World Health Organization developed the Safe Water System (SWS), which consists of water treatment at the point of use with a locally produced, dilute sodium hypochlorite solution, safe water storage, and behavior change techniques such as social marketing (*2*). The SWS has been shown to decrease diarrhea risk by 25%–85% (*3*–*7*) and has been implemented in >25 countries.

In November 2002, an SWS social marketing program was initiated in Malawi to prevent diarrheal illness among children <5 years of age, who were found to have a prevalence of diarrhea of 18% during a 2-week period in the 2000 Malawi Demographic and Health Survey (*8*). The SWS was promoted through radio announcements, flyers, signs on walls and minibuses, and billboards. The disinfectant solution, which was branded as WaterGuard, cost 10 kwacha (approximately $US 0.08) for sufficient solution to treat stored water for 1 month and was sold in small plastic bottles at supermarkets, pharmacies, and by street vendors.

In April 2005, CDC and the University of Malawi conducted a national household survey on healthcare, utilization patterns, and costs of childhood diarrhea and pneumonia in Malawi. We took the opportunity to measure mothers' awareness, perception, and reported use of WaterGuard.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Atlanta Research and Education Foundation, Decatur, Georgia, USA; ‡University of Malawi College of Medicine, Blantyre, Malawi; and §University of Liverpool, Liverpool, UK

The Study

This survey used the "modified segment" design described in the United Nation Children's Fund's End-Decade Multiple-Indicator Survey Handbook (*9*), which resulted in an equal-probability sample of 3,000 households in 30 enumeration areas throughout the country. All mothers of children <5 years of age were interviewed. Data were analyzed with SAS-callable SUDAAN 9.0.1 PROC RLOGIST (SAS Institute, Inc., Cary, NC, USA). Multivariate regression models were used to determine predictors of WaterGuard awareness and use. Colinearity and interactions between variables were assessed. To create an indicator of socioeconomic status, household asset factor scores, generated from a principal components analysis from the Malawi 1992 Demographic and Health Survey, were calculated by using the method described by Gwatkin et al. (*10*).

Among 3,000 households included in the survey, 1,787 mothers (or maternal caretakers) were identified, of whom 1,669 (93%) were eligible, having at least 1 child <5 years; all completed the survey. This sample was representative of the population distribution for Malawi, according to the Malawi 2004 Demographic and Health Survey (Table 1).

Among 1,669 mothers, 1,075 (64%; 95% confidence interval [CI] 58–71) had heard of WaterGuard; of these 726 (68%) believed the product was "to make water safe," 230 (21%) believed the product was "to prevent diarrhea," and 108 (10%) either did not know or gave another answer. Among the mothers who had heard of WaterGuard, 556 (52%) said they had used it "at some point in the past," and 124 (12%) reported that they were currently using it. Current users represented 7% (95% CI 4–11) of the total population. Among these, 77 (62%) said that WaterGuard caused "less diarrhea," or "less illness in the family." Rates of awareness and use of the product were higher among those living in an urban area than a rural area. Among 432 mothers who had used WaterGuard in the past, but were not using it at the time of the survey, 168 (39%) indicated that they "cannot afford it," 145 (34%) that it was "currently unavailable," 12 (3%) that they "don't like the taste," and 1 said she didn't "think it makes water safer." In all, 106 (25%) gave no reason for no longer using WaterGuard. In a multivariate model, WaterGuard awareness was independently associated with living in an urban area (adjusted odds ratio [aOR] 3.92 $p<0.001$), being a mother who had attended school (aOR 2.84, $p<0.001$), having a husband who had attended school (aOR 1.90, $p<0.001$), and higher wealth quintile (aOR 1.97 $p = 0.0003$) (Table 2). Current use of WaterGuard was independently associated with living in an urban residence compared to a rural residence (aOR = 2.01, $p = 0.0342$) (Table 2). The program budget

Table 1. Household description of mothers/caretakers interviewed and 2004 Malawi Demographic and Health Survey (DHS) data for comparison

Variable	2005 Household survey		2004 Malawi DHS, %
	No. (%)	95% CI*	
Region			
North	211 (13)	4–34	13
Central	725 (44)	26–63	41
South	716 (43)	26–63	46
Population			
Urban	156 (9)	3–27	18
Rural	1,489 (91)	73–97	82
Latrine			
Traditional pit toilet	1,290 (79)	75–87	79
No facility	289 (18)	13–25	16
Drinking water			
Improved source	1,117 (67)	53–79	64
Unimproved source	539 (32)	21–47	36

*CI, confidence interval.

and national WaterGuard sales in Malawi were substantially lower than comparable data from a similar SWS program in Zambia (Figure).

Conclusions

This national survey of Malawian mothers found awareness of WaterGuard to be high in a very poor country with limited commercial penetration into rural areas. In addition, over half of mothers who had heard of WaterGuard had tried it, and 12% of these mothers reported currently using the product at the time of the survey. Figures for awareness and past use in the present survey were consistent with an SWS survey conducted in Zambia (*11*), a country that borders Malawi and has a similar poverty and development ranking (*12*). However, reported current use of SWS in Zambia was, at 42%, substantially higher than in Malawi, reflecting substantially higher product sales in Zambia. Unlike the program in Malawi, which has had inconsistent and relatively low levels of funding, the SWS program in Zambia has had stable funding at a higher level and substantially greater sales. If the Malawi SWS program were able to obtain stable funding at higher levels, similar utilization rates to those in Zambia might be attainable.

A substantial gap exists between the percentage of mothers aware of WaterGuard who had tried it and those who were current users at the time of the survey (52% vs.

Table 2. Univariate and multivariate odds ratios and p value for awareness and current use of WaterGuard*

Predictor variable	Total	No. (%)	Crude OR (95% CI)†‡	Adjusted OR (95% CI)§	p value¶
Have heard of WaterGuard					
Urban population	156	147 (94.2)	9.87 (5.46–17.85)	3.92 (2.26–6.78)	<0.001
Mother attended school	909	713 (78.4)	3.75 (2.59–5.42)	2.84 (2.00–4.05)	<0.001
Husband attended school	1,072	776 (72.4)	2.89 (2.22–3.78)	1.90 (1.45–2.49)	<0.001
Higher wealth quintile	608	471 (77.5)	2.54 (1.69–3.80)	1.97 (1.41–2.74)	0.0003
Region					
Central	723	461 (63.8)	1.15 (0.47–2.80)	NS	
South	708	484 (68.4)	1.41 (0.63–3.14)	NS	
Mother employed	74	59 (79.7)	2.15 (1.03–4.50)	NS	
Improved drinking water	1,106	718 (64.9)	0.95 (0.61–1.47)	NS	
Currently using WaterGuard					
Urban population	144	34 (23.6)	2.86 (1.55–5.28)	2.01 (1.06–3.82)	0.0342
Region					
Central	456	67 (14.7)	3.36 (1.60–7.05)	1.88 (0.93–3.78)	0.1981
South	481	50 (10.4)	2.26 (0.96–5.31)	1.49 (0.60–3.70)	0.1981
Mother employed	58	13 (22.4)	2.22 (1.06–4.67)	1.67 (0.83–3.39)	0.1465
Higher wealth quintile	465	72 (15.5)	1.87 (1.08–3.23)	1.42 (0.84–2.42)	0.1866
Mother attended school	709	91 (12.8)	1.45 (0.69–3.01)	NS	
Husband attended school	771	93 (12.1)	1.22 (0.67–2.21)	NS	
Improved drinking water	712	87 (12.2)	1.22 (0.46–3.21)	NS	

*OR, odds ratio; CI, confidence interval; NS, variable did not meet criterion for remaining in the multivariate model.
†OR >1 for region central or region south indicates a higher probability than the north region.
‡OR >1 for higher quintile (4 and 5) indicates a higher probability than those in the lower quintile (1, 2, and 3).
§Predictor variables with a p = 0.10 in univariate analysis were included in the multivariate model to adjust for these simultaneously.
¶p value for Wald F statistic for the adjusted OR.

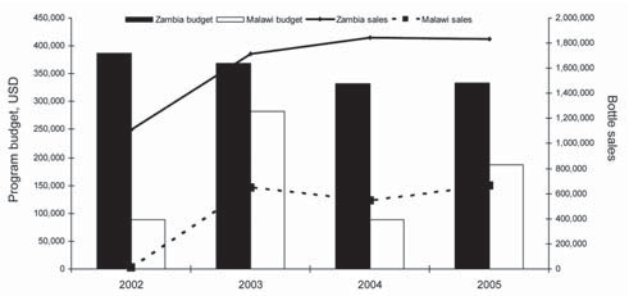

Figure. Annual program budget and product sales of Safe Water System programs in Malawi and Zambia, 2002–2005. Year 2005 total population: Zambia, 11,502,010; Malawi, 13,013,926 (www.cia.gov/cia/publications/factbook/geos/za.html). Budget and sales data provided by Population Services International. USD, US dollars.

12%, respectively), which can be considered a dropout rate of 78%. The goal of SWS in Malawi is to increase water quality in an area with limited access to clean water; therefore, sustained use among mothers is as important for long-term health effects as is increasing the initial use of the intervention. The reasons given by mothers who stopped using WaterGuard suggest that cost was a primary barrier to sustained use, especially among rural mothers. More research is needed to better define these and other reasons for discontinuation of water treatment to better inform efforts to increase WaterGuard availability and affordability. The positive perception of WaterGuard among those currently using it, together with the product's proven ability to disinfect water and prevent diarrhea, justifies continued efforts to market and evaluate the cost effectiveness of WaterGuard in Malawi (*13*).

Overall, findings of this survey support a need to increase WaterGuard promotion and distribution among poorer, less educated, and rural populations. Social marketing programs typically have difficulty reaching rural populations because of inadequate rural commercial infrastructure (*11*). If commercial mechanisms are not sufficient to promote rural use, then alternative, nontraditional approaches should be considered. For example, a program that used trained nurses in a maternal and child health clinic to promote SWS was associated with an increased rate of SWS use in rural Kenya (*14*), and use of motivational interviewing has resulted in higher purchase and usage rates of water disinfectant in Zambia (*15*). Using women's groups to market and sell products as income-generating activities, may also be efficacious (www.who.int/household_water/resources/freeman.pdf). We recommend that such marketing efforts be targeted to mothers who are least aware of the product and who could benefit the most from safe drinking water, including those who have not attended school, live in a rural area, or are have a lower socioeconomic status.

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Ms Stockman is an epidemiologist in the National Center for Immunization and Respiratory Diseases, CDC. Her interests include the epidemiology of enteric viruses and community-based public health interventions.

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Address for correspondence: Lauren J. Stockman, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop A34, Atlanta, GA 30333, USA; email: lstockman@cdc.gov

Blood Screening for Influenza

Michael Kai Hourfar,* Anna Themann,* Markus Eickmann,† Pilaipan Puthavathana,‡ Thomas Laue,§ Erhard Seifried,* and Michael Schmidt*

Influenza viruses, including highly pathogenic avian influenza virus (H5N1), could threaten blood safety. We analyzed 10,272 blood donor samples with a minipool nucleic acid amplication technique. Analytical sensitivity of the method was 804 geq/mL and 444 geq/mL for generic influenza primers and influenza (H5N1) subtype–specific primers. This study demonstrates that such screening for influenza viruses is feasible.

In the 20th century, 3 influenza-related pandemics occurred (1918 Spanish influenza, 1957 Asian influenza, and 1968 Hong Kong influenza) (*1*), which are now known to represent 3 different antigenic subtypes of the influenza A virus: H1N1, H2N2, and H3N2. Major influenza epidemics show neither periodicity nor a predictable pattern, and all differ from one another. Evidence suggests that true pandemics involving changes in hemagglutinin subtypes are caused by genetic reassortment in animal influenza A viruses. Since 2003, the World Health Organization has reported the infection of ≈218 persons and 124 deaths (56.9%; as of May 23, 2006) caused by the (H5N1) subtype in 10 different countries; a probable person-to-person transmission of the avian influenza virus was suggested (*2*). Most countries predicted death rates of 14–1,685 persons per 100,000 population in the event of a pandemic and estimated that up to 2,707 persons per 100,000 population would become infected (*3*).

Our study demonstrates that screening donor blood for influenza A (H5N1) subtype or for influenza viruses in general by minipool nucleic acid amplification technique (NAT) is feasible. To ensure the safety of blood products, this screening technique could be introduced into the blood-screening procedure without delay in the case of a pandemic.

The Study

To increase blood safety, we introduced minipool NAT screening in our blood donor service in 1997 for hepatitis B virus (HBV), hepatitis C virus (HCV), and HIV-1 and in 2000 for hepatitis A virus (HAV) and parvovirus B19 (*4*). For these purposes, 100-μL aliquots of up to 96 blood samples were pooled. The complete pool of up to 9.6 mL was centrifuged at 58,000× *g* for 60 min at 4°C. Viruses were extracted by using spin columns, and nucleic acid was eluted in a total volume of 75 μL. Only 60 μL of extract is needed for routine NAT screening. A residual volume of 15 μL can then be used for additional NAT testing (*5*) for influenza viruses.

The real-time quantitative amplification of influenza/H5 was performed according to the manufacturer's instructions (Artus Influenza/H5 LC RT-PCR Kit, QIAGEN, Hamburg, Germany) by using a thermocycler (LightCycler; Roche Applied Science, Mannheim, Germany). The test consists of 2 individual amplification reactions. In the first step, a generic influenza PCR is performed. The specificity of this reaction was demonstrated for all subtypes of influenza A (H1–H15, N1–N9) and all subtypes of influenza B. Samples with a positive test result in the first PCR were analyzed in a second PCR with influenza (H5N1)–specific primers and probes. Therefore, the assay allows differentiation between avian influenza (H5N1) and other influenza virus strains.

To mimic a situation like an H5-positive donation, a purified culture supernatant of Vero cells infected with influenza (H5N1) (strain A/Thailand/1 (KAN-1)/2004) (*6*) was used as an external quantification standard. Virion integrity in this preparation was confirmed by electron microscopy. The viral RNA concentration was determined in an external laboratory by multiple quantitative real-time PCR determinations (*7*). Different dilutions of the external influenza (H5N1) subtype quantification standard (0.0, 0.91, 1.96, 3.91, 7.81, 15.63, 31.25, 62.5, 125, and 250 PFU/mL) were prepared, and 100 μL of each dilution was spiked into 9.5-mL negative plasma pools. Each dilution was repeatedly spiked and tested in 8 minipools. Five microliters of the extract was analyzed with the generic influenza NAT as well as with the specific influenza (H5N1) NAT. Results are shown in Tables 1 and 2. Probit analysis of these data yielded a detection probability of >95% in parallel tests when an average of at least 13.4 PFU/mL (95% confidence interval [CI] 8.3–184 PFU/mL) and 7.4 PFU/mL (95% CI 5.2–14.7 PFU/mL) for influenza generic assay and for the influenza (H5N1)–specific test, respectively, were present in individual plasma samples before pooling.

A total of 117 routine minipools, representing 10,272 blood donor samples, containing an average of 88 ± 8 samples per pool, had previously been tested for HIV-1, HBV, HCV, HAV, and parvovirus B19. All pools were negative for influenza virus when tested with the generic influenza PCR and the influenza (H5N1)–specific PCR. One pool had invalid results (failed amplification of internal control RNA, representing 0.01% of all analyzed runs).

*German Red Cross, Frankfurt, Germany; †Phillips University, Marburg, Germany; ‡Mahidol University, Bangkok, Thailand; and §QIAGEN, Hamburg, Germany

Table 1. Analytical sensitivity for influenza virus in plasma samples*

PFU (H5N1) spiked in minipools	No. positive/ no. tested	% Positive
250.0	8/8	100
125.0	8/8	100
62.5	8/8	100
31.3	8/8	100
15.6	7/8	87.5
7.8	8/8	100
3.9	4/8	50
0.0	0/8	0

*Influenza (H5N1) standard was extracted from 9.6 mL of 96 pooled donor samples after centrifugation. Five microliters of 75-µL nucleic acid extract was analyzed. The 95% detection limit was 13.4 PFU/mL; the 50% detection limit was 4.8 PFU/mL.

Conclusions

As reported by AuBuchon et al. (*8*), NAT significantly increased the safety of blood products. At the German Red Cross, look-back examinations showed only 1 transfusion had transmitted HIV-1 (1998) after the introduction of NAT testing. Blood donor screening by NAT was made technically and financially feasible by creating minipools of up to 96 individual samples per pool. Roth et al. demonstrated an efficient enrichment for all tested viruses in plasma samples (*9*). In the absence of an infective donor, different concentrations of the new influenza genotype H5N1 were spiked into minipools of 95 samples. As shown in Table 2, the influenza (H5N1) subtype was detected by the generic influenza primers as well as by the influenza (H5N1)–specific primers when our routine minipool screening procedure was used. Sensitivity was expressed as PFU/mL and can be converted into viral genome copy number according the calculation of Yoshikawa et al. (*7*). Therefore, the analytical sensitivity was ≈804 geq/mL and 444 geq/mL for a generic influenza and for the influenza (H5N1) subtype, respectively.

After screening 10,272 samples by minipool NAT, none of the samples were found to be infected by influenza, which corresponds with the low EISS Index (European Influenza Surveillance Scheme index) of <20 during the study period (February–April, 2006) (*10*). An EISS index >80 is expected during an influenza epidemic, as was seen in 2005. Therefore, blood screening should be repeated during the next acute influenza season.

Table 2. Analytical sensitivity for avian influenza (H5N1) virus subtype for plasma samples*

PFU (H5N1) spiked in minipools	No. positive/ no. tested	% Positive
125.0	8/8	100
62.5	8/8	100
31.3	8/8	100
15.6	8/8	100
7.8	7/8	87.5
3.9	7/8	87.5
1.9	4/8	50
0.9	3/8	37.5
0.0	0/8	0

*Influenza (H5N1) standard was extracted from 9.6 mL of 96 pooled donor samples after centrifugation. Five microliters of 75-µL nucleic acid extract was analyzed. The 95% detection limit was 7.4 PFU/mL; the 50% detection limit was 2.5 PFU/mL.

Accepted incubation periods for influenza range from 2 to 10 days (*11*,*12*). As with other viruses, a viremic phase of infection can be assumed to precede clinical symptoms such as fever (*13*,*14*). Recently Chutinimitkul et al. (*15*) detected influenza (H5N1) virus (3,080 copies/mL) in the plasma of a 5-year-old boy, which indicates a viremic phase of influenza (H5N1) infection. Those donors may be infective, especially to immunosuppressed patients. In addition to quarantine of infected patients, treatment with antiviral drugs, and development of avian influenza vaccines, blood donors should be tested during a pandemic to avoid transfusion-transmitted infections. Our study demonstrates that NAT screening could be incorporated into blood testing without delay and that the influenza virus could be sufficiently enriched by centrifugation. Sensitivity of our influenza-screening method would have been sufficient to detect recently reported virus concentrations in plasma of infected persons (*15*). However, as with all minipool methods, infections can be transmitted to transfusion recipients on rare occasions because the viremia level in the donor is below the analytical sensitivity of the screening assay.

To reduce this risk, a selective infectious dose NAT strategy (e.g., triggering of infectious dose NAT testing when at least 1 viremic donation is collected per week with the standard minipool screening algorithm), as performed for West Nile virus (WNV) screening in the United States might be necessary. Implementation of WNV-NAT in the United States in 2003 interdicted well over 1,000 donations from persons infected with WNV and is a good example of successful implementation of NAT screening for emerging viruses.

The collective fight against new viruses such as severe acute respiratory syndrome virus, WNV, or influenza (H5N1) presents an immense challenge for the whole community, but new molecular-biologic methods offer opportunities to overcome this challenge. NAT screening tests are now available soon after the sequencing of new viruses. In the absence of a general pathogen inactivation method for all blood products (erythrocytes, platelets, and plasma), the NAT screening procedure allows testing for new viruses to ensure blood safety.

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Mr Hourfar is a research scientist at the German Red Cross blood donor service. His research interests include the molecular epidemiology of pathogens with special focus on blood safety.

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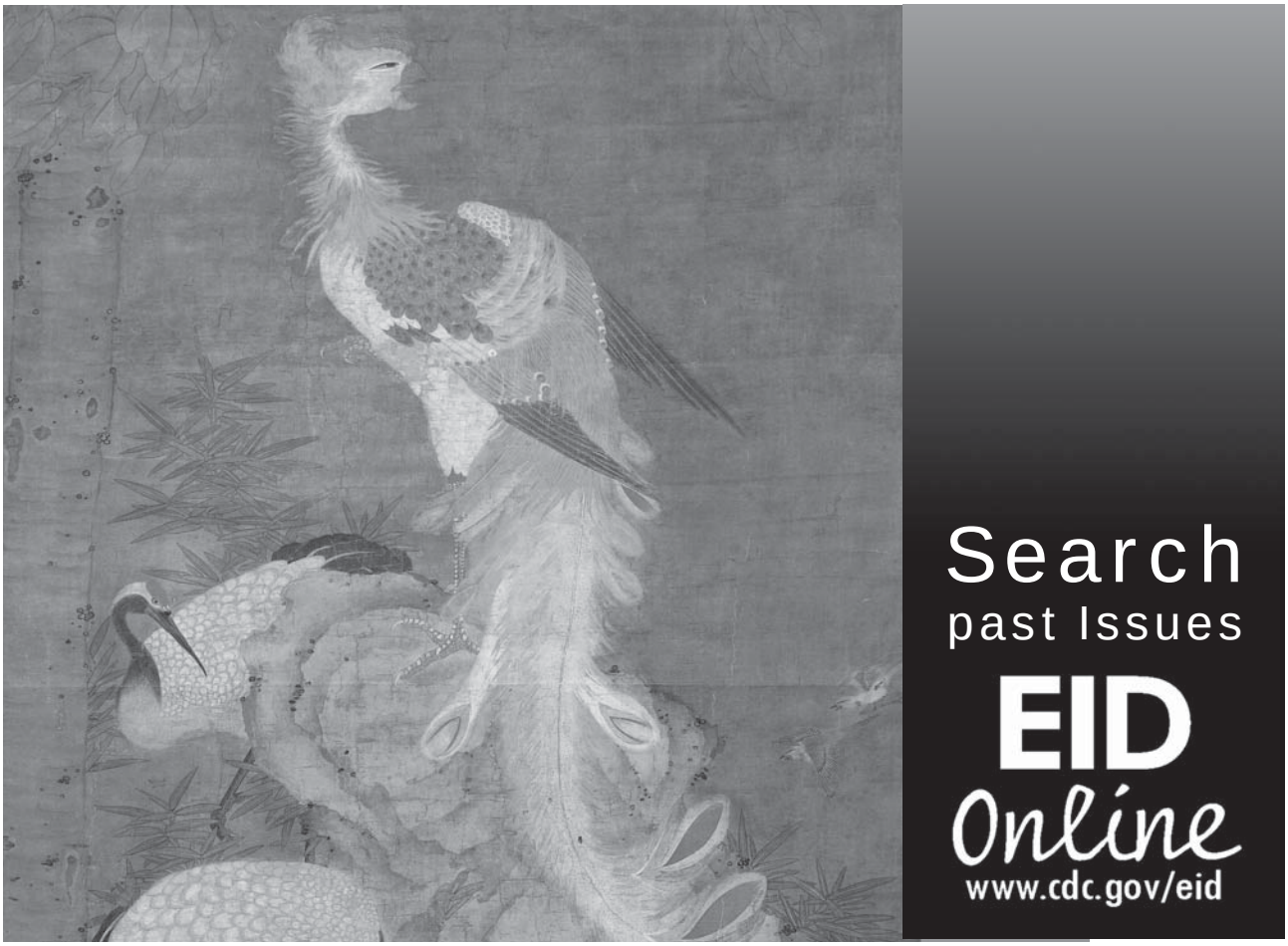
Address for correspondence: Michael Schmidt, Institute of Transfusion Medicine and Immunohematology, Johann Wolfgang Goethe University, German Red Cross, Sandhofstr. 1, 60528 Frankfurt am Main; Frankfurt, Germany; email: mschmidt@blutspende.de



Fatal Coxsackievirus A-16 Pneumonitis in Adult

François Legay,*[1] Nicolas Lévêque,† Arnaud Gacouin,* Pierre Tattevin,* Julien Bouet,* Rémi Thomas,* and Jean-Jacques Chomel†

Coxsackievirus A-16 (CVA-16) is the agent of hand, foot, and mouth disease in children. We report a case of fatal pneumonitis in an adult due to a CVA-16 strain with a low (78.6%) rate of sequence homology with the reference strain. A modified, more virulent, strain of CVA-16 could be emerging.

Hand, foot, and mouth disease (HFMD) is a benign condition caused by coxsackievirus A type 16 (CVA-16), which affects young children and usually resolves uneventfully. Rarely, it may be associated with complications such as meningitis, encephalitis, myelitis, and respiratory failure. During outbreaks of enterovirus infection, respiratory failure has been associated with cardiac failure in children infected with enterovirus 71 (EV 71) but not in those infected with CVA-16 (*1*,*2*). We report a case of CVA-16 pneumonitis that was fatal for an adult.

The Case

In April 2006, a 76-year-old man was admitted to the emergency department of Pontchaillou University Hospital, Rennes, France, with acute onset of fever, lumbar pain, and dyspnea. Examination found a temperature of 37.9°C and bilateral pulmonary crackles. Laboratory results were the following: leukocytes 9,600 cells $\times 10^6$/L (90.6% neutrophils), C-reactive protein 216 mg/L (normal value <5 mg/L), and arterial oxygen partial pressure 67 mm Hg (room air). Chest radiograph was unremarkable, and *Legionella pneumophila* urinary antigen was not found. The patient was treated with amoxicillin-clavulanate and ofloxacin. On day 3, acute respiratory distress syndrome developed, and the patient required mechanical ventilation. Computed tomographic scan of the thorax showed bilateral alveolointerstitial infiltrates (Figure). Transthoracic echocardiograph and pulmonary artery catheterization showed normal left ventricular function. Serum troponin levels were within normal limits.

A bronchoalveolar lavage (BAL) was performed; no pyogenic bacteria, *L. pneumophila*, *Mycobacterium tuberculosis*, *Pneumocystis jiroveci,* or *Aspergillus* sp. were isolated despite appropriate staining for direct examination and cultures on appropriate media. Negative results were obtained in *M. tuberculosis* PCR and immunofluorescence assays (IFAs) for *P. jiroveci* and *L. pneumophila*. The result of a PCR for human herpesviruses, which used herpes consensus identification, was negative. Results of testing for respiratory syncytial virus, influenza virus, parainfluenza virus, and adenovirus by IFA and ELISA, which used specific monoclonal antibody, were all negative. In addition, serologic test results for *L. pneumophila*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *C. psittaci*, *Coxiella burnetti,* and HIV were negative. Three sets of blood cultures taken at the time of hospital admission remained sterile. Test results for antinuclear factors, antiglomerular basement-membrane antibodies, and antineutrophil cytoplasmic antibodies were negative.

BAL was positive in 2 enteroviral PCR assays that used EV1 primer from Rotbart et al. (*3*) and real-time PCR with primers and probe adapted from Verstrepen et al. (*4*). In addition, on day 3, MRC-5 cell culture showed a specific cytopathic effect, which was confirmed as enterovirus by indirect IFA that used enterovirus mouse monoclonal antibody (Novocastra, Newcastle, UK) and fluorescein isothiocyanate–conjugated AffiniPure Goat Anti-Mouse (Jackson Immuno-Research, West Grove, PA, USA). Enterovirus was also detected in serum and pharyngeal samples by real-time PCR. Results of real-time PCR and cell culture (MRC-5 and LLC-MK2) were also positive for enterovirus on BAL performed on days 8 and 14. Kinetics analysis in real-time PCR showed a 100-fold decrease in viral load by comparison of cycle thresholds between day 8 and day 14 BAL. Serologic testing for enterovirus showed an 8-fold increase in enterovirus antibody titration by complement fixation between days 3 and 11. Immunoglobulin M to echovirus/coxsackievirus was detected by ELISA in serum on day 11 (Genzyme, Virotech, Chilly Mazarin, France).

No drug is currently approved for the treatment of enterovirus infection. Pleconaril may be of value in severe enteroviral infections (*5*) but is no longer available because licensure was not pursued. The patient did not improve and died on day 28 of intractable hypoxemia. Histologic examination of postmortem pulmonary biopsy specimen showed diffuse alveolar damage and fibrosis, real-time PCR detected enterovirus, and viral cultures were negative. The enterovirus isolated on the day-3 BAL was identified as CVA-16 by partial sequencing of the VP1 region that encompasses the BC loop. This region was amplified with primers 292 as sense primer and 222 as antisense primer

*Centre Hospitalier Universitaire Pontchaillou, Rennes, France; and †Centre Hospitalier Universitaire Edouard Herriot, Lyon, France

[1]Current affiliation: Centre Hospitalier Saint-Brieuc, Saint-Brieuc, France

(*6*). The sequences obtained (reference no. bankit 845096-DQ993173 until definitive number assigned) were aligned with the corresponding region in GenBank. Comparison of a 338-nt sequence with that of the CVA-16 reference strain (prototype BrCr) and the EV 71 reference strain (prototype G-10) showed nucleotide identity rates of 78.6% and 64.6%, respectively.

On subsequent questioning, the patient's wife reported that he had had close contact with his granddaughter, who had active HFMD, while in the United Kingdom during the week before he was admitted. Although a large HFMD outbreak occurred in the United Kingdom in 1994 (*7*), no enterovirus outbreaks had been reported at the time of the patient's admission. During the 1994 UK outbreak, CVA was isolated in 28 of 40 patients (type CVA-16 in 21 patients and CVA-10 in 7 patients), but secondary cases in family members were rare and no case of pneumonitis or death was reported.

Conclusions

Fatal CVA-16 infection has been described infrequently in children who had HFMD associated with myocarditis (*8*). We report a fatal CVA-16 infection associated with pneumonitis in an adult; to our knowledge, this is the first such report. Our patient had neither myocarditis nor left ventricular dysfunction, as demonstrated by pulmonary artery catheterization results, echocardiograph results, and serum troponin levels. In 2003, 7 fatal cases of HFMD in children were reported in Singapore (*9*). These children had interstitial pneumonitis, either alone or associated with myocarditis or encephalitis. EV 71 was isolated in 4 cases and echovirus 16 in 1. The CVA-16 strain isolated from our patient had a low percentage of nucleotide identity with the reference strain (78.6%); a threshold of 90% is usually required to define strain homology. This may be a sign that this virus is evolving. A strain similar to that from our patient was circulating in China from 1999 through 2004 (98% nucleotide identity; GenBank accession no. AY821798) and was isolated from fecal samples of children with HFMD or suspected enterovirus infection (*10*). This strain was associated with local yearly outbreaks in which only a few cases of neurologic disease and no deaths were reported. According to phylogenetic analysis based on VP4 207-bp nucleotide sequence, the authors concluded that 3 genetic lineages were circulating in Asia at that time and suggested that the same tendency may apply in other continents (*10*).

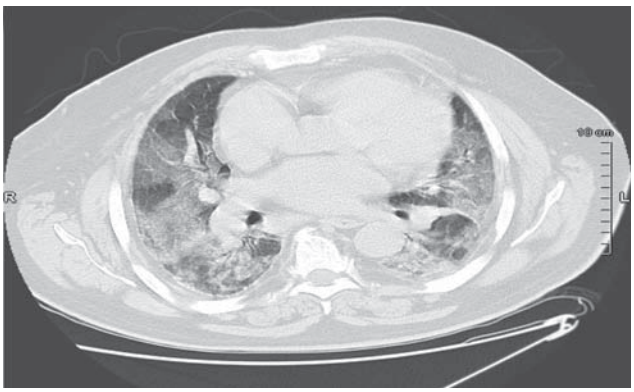


Figure. Thoracic computed tomographic scan on day 3, showing bilateral alveolo-interstitial infiltrates.

We report what we believe to be the first case of CVA-16 pneumonitis in an adult, with fatal outcome. Preliminary sequence analysis revealed a low rate of homology between the CVA-16 strain we isolated and those previously published, which suggests that a new, more virulent, strain of CVA-16 could be emerging. To compare the sequences to those published in GenBank, we are sequencing the complete part of the genome encoding the VP1 capsid protein.

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Dr Legay works in the intensive care unit, Centre Hospitalier Saint-Brieuc, France. His major research interests include emerging infectious diseases, severe pneumonitis, and septic shock.

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Address for correspondence: Pierre Tattevin, Service de Maladies Infectieuses et Réanimation Médicale, Pontchaillou University Hospital, Rue Le Guilloux, 35033 Rennes CEDEX, France; email: pierre.tattevin@chu-rennes.fr

Estimating Severe Coccidioidomycosis in California

Valerie J. Flaherman,* Richard Hector,*† and George W. Rutherford*†

We used hospital discharge data to estimate incidence and distribution of coccidioidomycosis-associated hospitalizations in California. For 1997–2002, the average annual rate of hospitalization was 3.67 per 100,000 population. County of residence, older age, black race, male sex, HIV infection, and pregnancy were strongly associated with increased risk for hospitalization.

Coccidioidomycosis is caused by the fungi *Coccidioides immitis* and *C. posadasii,* which are present in soil in disease-endemic areas. Data are sparse on frequency and incidence of severe coccidioidomycosis that requires hospitalization. In California, population increase, changing racial composition, and increasing numbers of immunocompromised persons may have affected the incidence and severity of disease in recent years. We used hospital discharge data to estimate frequency and incidence of hospitalization with coccidioidomycosis for California, including its counties and its demographic subgroups.

The Study

We examined the Inpatient Hospital Discharge Data Set from the California Office of Statewide Health Planning and Development for 1997–2002 (*1*), which contains inpatient discharge diagnoses from all nonfederal hospitals in California. We abstracted all records with any International Classification of Diseases, 9th edition (ICD-9), code for coccidioidomycosis (114–114.5 and 114.9, with 114.2 representing coccidioidal meningitis) and defined each record as a discrete hospitalization.

From each record, we extracted year of admission, county of residence, age, race, ethnicity, sex, presence of HIV infection (ICD-9 codes 042 or V08), pregnancy status (ICD9 codes V22-V23.9 or 630–676.9), vital status at discharge, and record locator number (RLN) (available for 1997–2000 only). We obtained population estimates from California Department of Finance by county of residence, age, racial-ethnic group, and sex (*2*,*3*). Numbers of AIDS cases and estimates of the population with HIV were obtained from the California Department of Health Services (*4*).

All statistical analysis was conducted by using Stata 8.2 (Stata Corp., College Station, TX, USA). We calculated the frequency of hospitalization by county, age group, racial-ethnic group, sex, pregnancy status, and immune status. Incidence of initial hospitalization for severe coccidioidomycosis was estimated by using each earliest hospitalization with a given RLN during the years 1997–2000 for which RLN was available, and rates of repeat hospitalization were calculated on the basis of subsequent hospitalizations with the same RLN. Mortality rate was calculated as crude incidence rate for death among those hospitalized for coccidioidomycosis with unique RLNs.

Bivariate relative risks (RRs) were calculated for the effect of pregnancy status and immune status on the frequency of hospitalization. Multivariate Poisson regression was used to estimate RR of year, county, age, racial-ethnic category, and sex on the frequency of hospitalization for coccidioidomcyosis. Among those hospitalized, multivariate logistic regression was used to evaluate odds ratios (ORs) for race, ethnicity, and sex on rehospitalization and meningitis.

Records for which data were missing for age, sex, race/ethnicity, or county were not included in multivariate analysis. Records that lacked data for county were not included in crude incidence calculations by county. For crude incidence calculations by age, race/ethnicity, and sex, cases for which category was unknown were redistributed among known categories in the same proportion as they occurred among those with known category. This study was approved by the Committee on Human Research, University of California, San Francisco.

From 1997 through 2002, of 7,457 inpatient hospitalizations associated with coccidioidomycosis in nonfederal institutions in California, 3,707 (50%) had a principal diagnosis of coccidioidomycosis, 1,605 (22%) had a first additional diagnosis of coccidioidomycosis, and 896 (12%) had a second additional diagnosis of coccidioidomycosis. Frequency of hospitalization for coccidioidomycosis was 3.7 per 100,000 residents per year (Table 1). Kern, Los Angeles, and San Diego counties had highest total number of hospitalizations and together accounted for 47% of all hospitalizations. There were 417 deaths, resulting in a mortality rate of 2.1 per 1 million California residents annually.

For years for which an RLN was available (1997–2000), 63% of hospitalizations were initial and 37% were repeat. The incidence of initial hospitalization for severe coccidioidomycosis was 2.4 per 100,000 residents, and 8.9% of persons initially hospitalized with coccidioidomycosis died in the initial or a subsequent hospitalization.

Pregnant women were more likely than nonpregnant women to be hospitalized with a code for coccidioidomycosis (RR 2.5, 95% confidence interval [CI] 2.03–3.08). Compared with all Californians, RR for hospitalization for persons with AIDS was 34.5 (CI 31.0–38.4) and for persons with HIV was 13.9 (CI 12.5–15.5). When only records

*University of California, San Francisco, California, USA; and †California State University, Bakersfield, California, USA

Table 1. Hospitalizations for coccidioidomycosis, California, 1997–2002

Category	Total hospitalizations	Total person-years (x 10^6)	Frequency of hospitalization*	Frequency of hospitalization for coccidioidal meningitis*
Total	7,457	203.0	3.67	0.657
Year				
1997	1,269	32.5	3.90	0.706
1998	1,144	32.9	3.50	0.706
1999	1,167	33.4	3.5	0.61
2000	1,100	34.0	3.23	0.62
2001	1,291	34.7	3.7	0.58
2002	1,486	35.3	4.2	0.71
Highest incidence counties				
Kern	1,700	3.97	42.8	
Tulare	479	2.21	21.7	
Kings	133	0.77	17.4	
San Luis Obispo	170	1.48	11.5	

*Per 100,000 residents per year.

with RLNs were examined, 24% of persons admitted with coccidioidomycosis who had HIV coinfection died during hospitalization, compared with 8.2% of persons admitted with coccidioidomycosis who did not have HIV coinfection ($p<0.005$ by χ^2 analysis).

In multivariate Poisson regression that used California Department of Finance population estimates, older age, black race/ethnicity, and male sex were associated with increased risk for hospitalization. Native American and Hispanic race/ethnicity was protective for this outcome (see reference groups in Table 2). Asian-Pacific Islander race/ethnicity was protective on a statewide level but was a risk factor in the 4 counties with the highest incidence.

Logistic regression showed that black persons hospitalized with a diagnosis of coccidioidomycosis had increased risk for rehospitalization (OR 2.08, CI 1.59–2.73) compared with white persons, controlling for year, county, age, and sex. Controlling for the same confounders, Asian-Pacific Islanders hospitalized with coccidioidomycosis had increased risk for meningitis (OR 1.63, CI 1.02–2.63); Hispanic race/ethnicity was protective against meningitis (OR 0.63, CI 0.48–0.84).

Conclusions

Hospitalizations for coccidioidomycosis are common in California, especially in disease-endemic areas. Deaths from coccidioidomycosis average ≈70 per year statewide. Persons with AIDS have both a very high frequency of hospitalization for coccidioidomycosis and a very high proportion of deaths from the disease. Persons with AIDS in the 4 counties with the highest frequency of coccidioidomycosis (Kern, Tulare, Kings, and San Luis Obispo) have a frequency of hospitalization for coccidioidomycosis that approaches 1% per year. This study confirms several well-known risk factors for coccidioidomycosis, including black race, middle age and older age, and pregnancy (*7*). We did not find evidence supporting previous reports of Hispanic and Asian racial/ethnic background as a risk factor for coccidioidomycosis hospitalization statewide (*8*,*9*).

This study has several limitations. First, we included all hospitalizations that contained any discharge diagnosis of coccidioidomycosis; 73% of our included hospitalizations coded coccidioidomycosis as principal or first additional diagnosis. Among those hospitalizations for which coccidioidomycosis was not principal or first additional diagnosis, it is unclear whether other principal diagnoses (such as AIDS) would have caused hospitalization in the absence of coccidioidomycosis. In this respect, our findings may overestimate the incidence of disease. Second, we have no information about persons hospitalized in federal hospitals, which could lead to an underestimation of effects of disease or bias of results regarding disease distribution. However, because only 19 of 570 hospitals licensed in California in 1999 were federal, the bias of omitting federal hospitals is likely to be small (*10*). Third, we had information on duplicate hospitalizations for 1997–2000 only, and some persons either may have had a repeat hospitalization subsequently or may have been hospitalized for coccidioidomycosis before this period. Fourth, we have no data on the subcategory of persons with Asian ancestry. The increased risk we found for Asian-Pacific Islanders in the 4 counties with highest incidence is not consistent with risk statewide, and this might be partly explained by differential risk by subcategory of race/ethnicity. However, differential exposure patterns based on employment or recreation might also contribute to the discrepancy. Further research in this area is needed.

The risk for severe disease and death attributable to coccidioidomycosis in California is of a magnitude similar to the risk from varicella in the state before the varicella vaccine (*11*,*12*). Furthermore, a substantially higher risk exists for many subgroups, including residents of high-incidence counties, middle-aged and older persons, pregnant women, black residents, and those with HIV infection. Recent progress in vaccine development has raised the

Table 2. Frequency and relative risk for hospitalization for coccidioidomycosis in California and its 4 highest-incidence counties, 1997–2002*

Population	Frequency of hospitalization†	RR for discharge diagnosis of coccidioidomycosis	Frequency of hospitalization in highest incidence counties†	RR for discharge diagnosis of coccidioidomycosis in highest incidence counties
Race‡				
White	3.6	Ref	26.6	Ref
Hispanic	3.4	0.73 (0.68–0.78)	24.1	0.71 (0.63–0.79)
Black	8	2.68 (2.48–2.91)	80.8	2.43 (2.10–2.82)
Native American	1.4	0.32 (0.21–0.51)	12.7	0.37 (0.20–0.70)
Asian-Pacific Islander	2	0.78 (0.70–0.87)	51	1.62 (1.34–1.97)
Sex‡				
Female	2.3	Ref	36.6	Ref
Male	5	2.14 (2.03–2.27)	21.9	1.67 (1.53–1.85)
Age, y‡				
≤14	0.5	0.12 (0.10–0.14)	3.9	0.12 (0.09–0.15)
15–49	3.5	Ref	31.3	Ref
50–69	7.1	2.13 (2.01–2.26)	57.2	1.83 (1.66–2.03)
≥70	7.3	2.74 (2.54–2.97)	47.0	1.83 (1.58, 2.12)
Special conditions§				
Pregnancy¶	3.8	2.5 (2.03–3.08)	51.9	
AIDS	127	34.5 (31.0–38.4)	912	31.0 (23.8–40.4)
All HIV	51	13.9 (12.5–15.5)	319	10.8 (8.3–14.1)

*Multivariate results reported for 6,465 cases with no missing data (87%). RR, relative risk; Ref, referent. Values in parentheses are 95% confidence intervals.
†Crude incidence per 100,000 residents in Kern, Tulare, Kings, and San Luis Opispo counties.
‡RR by multivariate Poisson model controlling for year, county, age, race, and sex. For the years 1997–1999, racial-ethnic categories were White, Hispanic, Black, Native American, and Asian-Pacific Islander. For the years 2000–2002, racial-ethnic categories were White, Hispanic, Black, Native American, Asian, Pacific Islander, and multirace. The population of multirace represented ≈1% of populations relevant to the study and was not included in the analysis. California Department of Finance population estimates from 2000–2002 for Asians and Pacific Islanders were combined into a single category, Asian-Pacific Islander, to match coding of the Office of Statewide Health Planning and Development database. All Hispanics in the Department of Finance data were assumed to be of white race.
§Bivariate relative risk.
¶To estimate a denominator for the pregnant population, we estimated the total person-years of pregnancy for each county in California in the following manner. The total number of live births was multiplied by 0.75 (to approximate 9 mo of pregnancy) and added to the total number of fetal deaths, multiplied by 0.56 (to estimate a gestation of 30 weeks). This sum was finally added to the total number of abortions, multiplied by 0.19 (to estimate a gestation of 10 weeks). Estimates of live births and fetal deaths were obtained from the Center for Health Statistics Birth Rate Tables (*5*). Annual number of abortions was estimated from federal abortion surveillance data from 1997 (*6*).

possibility of a way to better control this disease (*13*). The development of new therapeutic and preventive modalities could do much for the population at risk and should be considered a priority for healthcare research. Our data offer a good overall estimate of the incidence of severe disease for the state, but to assess the success of a new vaccine, further studies will need to determine initial hospitalization and primary cause of hospitalization with greater specificity.

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Dr Flaherman is a general pediatrics fellow at the University of California, San Francisco. Her research interests are public health epidemiology in California and newborn preventive medicine.

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Address for correspondence: Valerie J. Flaherman, Department of Pediatrics, University of California, San Francisco, Box 0503, San Francisco, CA 94143-0503, USA: email: flahermanv@peds.ucsf.edu

Canine-Origin G3P[3] Rotavirus Strain in Child with Acute Gastroenteritis

Simona De Grazia,* Vito Martella,† Giovanni M. Giammanco,* Miren Iturriza Gòmara,‡ Stefania Ramirez,* Antonio Cascio,§ Claudia Colomba,* and Serenella Arista*

Infection by an animal-like strain of rotavirus (PA260/97) was diagnosed in a child with gastroenteritis in Palermo, Italy, in 1997. Sequence analysis of VP7, VP4, VP6, and NSP4 genes showed resemblance to a G3P[3] canine strain identified in Italy in 1996. Dogs are a potential source of human viral pathogens.

Group A rotaviruses are enteric pathogens of humans and animals. Rotaviruses usually exhibit host species restriction, although interspecies transmission or reassortment between animals and humans viruses can occur (*1*). Sequence analysis of the genes that code for the 2 outer capsid proteins VP7 and VP4, for the inner capsid protein VP6, and for the nonstructural protein NSP4 is useful for gathering epidemiologic information and tracing the origin of unusual rotavirus strains. To date, 15 VP7 genotypes (G types 1–15), 27 VP4 genotypes (P types [1]–[27]), 4 VP6 subgroup specificities (SGs I, II, I+II, and nonI/nonII), and 5 NSP4 genotypes (A–E) have been established in human and animal group A rotaviruses (*1*,*2*). Human rotaviruses usually exhibit G1, G3, G4, and G9 types in association with P[8] type, SGII specificity, and NSP4 B type; G2 rotaviruses are more often associated with P[4] type, SGI specificity, and NSP4 A type (*1*). By polyacrylamide gel electrophoresis (PAGE), most animal SGI and SGII and human SGII rotavirus strains display a "long" pattern of migration (e-type) of the 11 dsRNA genomic segments; almost all SGI human rotavirus strains possess a "short" e-type (*1*).

A number of strains with unusual VP7 and VP4 genes, regarded as animal-like, have been sporadically identified in humans and have acquired epidemiologic relevance in some geographic areas (*3*). Dogs are regarded as vectors of viral, bacterial, or parasitic zoonoses for persons of all ages, but risks for transmission of enteric viruses are almost ignored. However, early in the study of rotavirus epidemiology, symptomatic and asymptomatic infections by canine/feline-like rotavirus strains (HCR3A, HCR3B, Ro1845), characterized as G3P5A[3], long e-type and SGI, were identified in young children (*4*,*5*).

*University of Palermo, Palermo, Italy; †University of Bari, Valenzano, Bari, Italy; ‡Health Protection Agency, London, UK; and §University of Messina, Messina, Italy

The Study

In February 1997, rotavirus infection was diagnosed (by PAGE analysis) in a 2-year-old child hospitalized with severe acute diarrhea at the "G. Di Cristina" Children's Hospital of Palermo. The virus, PA260/97, exhibited a long e-type and was recognized by an SG-specific monoclonal antibody (MAb) and by a VP7-specific MAb as SGI and G3 (*6*). Accordingly, strain PA260/97 displayed a genetic/antigenic constellation that is usually observed in animal-like viruses. For confirmation of the initial antigenic characterization and information about the VP4 (P) genotype, strain PA260/97 was characterized at the molecular level. By PCR genotyping of the VP7 and VP4 genes with panels of primers specific for various human G and P types (*3*,*7*,*8*), the VP7 was characterized as G3 and the VP4 was untypeable. To characterize strain PA260/97 in more detail, we determined the sequence of the VP7, VP4 (VP8*), VP6, and NSP4 genes. We also determined the VP7, NSP4, and VP6 sequences of human strain PAH101/97 (G3P[8], SGII, long e-type), detected in Palermo in the same year, as well as the sequences of the VP8*, VP6, and NSP4 genes of 2 G3P[3], SGI, long e-type strains, RV 198/95, and RV 52/96, isolated from dogs in Italy in 1995 and 1996, respectively (*9*).

The sequences of human strains PA260/97 and PAH101/97 and of canine strains RV198/95 and RV52/96 have been deposited in GenBank. The accession numbers are as follows: EF442738 (VP6), EF442733 (VP7), EF442735 (VP8*), and EF442741 (NSP4) for strain PA260/97; EF534715 (VP6), EF442734 (VP7), and EF534716 (NSP4) for strain PAH101/97; EF442737 (VP6), EF442736 (VP8*), and EF442739 (NSP4) for strain RV198/95; EF442742 (VP6), EF442740 (VP8*), and EF442743 (NSP4) for strain RV52/96.

The VP8* of strain PA260/97 displayed the highest amino acid (aa) identity (98%) to the canine strain RV52/96, G3P[3], isolated in Italy in 1996 (Figure 1). Similar, the VP7 of strain PA260/97 displayed the highest identity to G3 rotaviruses, with the best match (99% nt and aa) to the canine strain RV52/96; identity to reference human G3 strains (YO, AU-1, Ma09004, TK28) and to the human G3 strain PAH101/97, isolated in Palermo in 1997, ranged from 77% to 78% nt and from 88% to 90% aa. In the phylogenetic VP7-based analysis (Figure 2), human strain PA260/97 clustered with animal G3 strains. Species-specific patterns have been demonstrated in the VP7 gene of G3 human and animal rotaviruses (*9*). These patterns are sug-

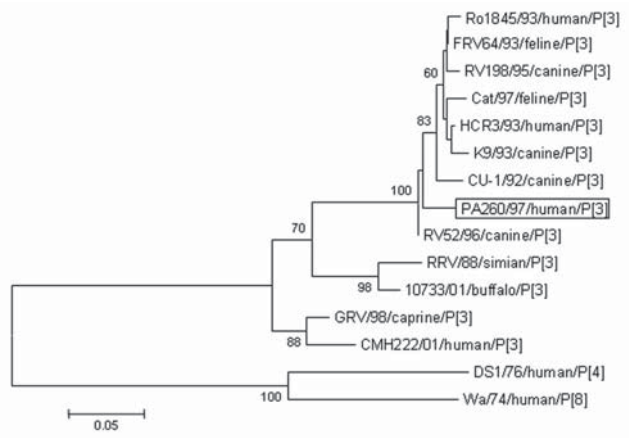

Figure 1. Phylogenetic analysis of the deduced amino acid sequence derived from VP4 gene of the PA260/97 human rotavirus strain and other P[3] rotavirus strains. The tree was generated by the neighbor-joining method using the ClustalW program (http://dambe.bio.uottawa.ca/dambe.asp). Scale bar indicates nucleotide substitutions (×100).

gestive of mechanisms of host-species restriction and are useful for tracing the origin of unusual rotavirus strains.

A close genetic relationship between human strain PA260/97 and canine strain RV52/96 was also observed by comparing the genes that encode VP6 and NSP4. On the basis of MAb reactivity, strain PA260/97 was characterized as SGI and strain PAH101/97 was characterized as SGII. Sequencing of an informative fragment of the VP6 gene (*10*) showed a close genetic relationship (97% aa) between strain PA260/97 and canine strain RV52/96 and only 86% aa identity with strain PAH101/97. Sequence analyses of NSP4 enabled characterization of strains PA260/97, RV198/95, and RV52/96 into the NSP4 genotype C; strain PAH101/97 (G3P[8]) was characterized as NSP4 B genotype. The highest identity was observed between strains PA260/97 and RV52/96 (98% nt and 99% aa).

Conclusions

Rotavirus strains with a G3P[3] combination are usually detected in cats and dogs. Despite only a few reports of rotavirus isolation from dogs with gastroenteritis, all canine strains identified thus far in the United States, Japan, and Europe (CU-1, A79-10, LSU79C-36, RS15, RV198/95, and RV52/96) display G3 and P[3] specificities (*9*). More recently, G3P[3] rotaviruses have also been identified in monkeys and goats (*1*,*11*). In contrast, detection of G3P[3] in humans is uncommon; only 4 G3P[3] strains—HCR3A, HCR3B (*4*,*12*), Ro1845 (*5*), and CMH222 (*13*)—have been reported. By sequence analysis and by RNA-RNA hybridization, strains HCR3A and Ro1845 were found to be related to canine and feline rotaviruses rather than to human G3 rotaviruses; strain CMH222 appeared to be genetically related to both simian and caprine G3P[3] rotaviruses (*13*). Genetic reassortment between human and canine rotaviruses may also have occurred. The Mexican rotavirus strain 7177-1042 bears a common human VP4 gene, P[8], in conjunction with a canine-like VP7 gene, closely related to the VP7 of canine strain RV198/95 (*14*).

Canine rotavirus infection is considered a minor disease in young dogs (pups) because it is usually mild or unapparent; however, serologic investigations have shown a high prevalence of antibodies to rotavirus in adult dogs (*15*). Previous documented examples of infections in humans by canine-like rotavirus strains have been associated with either asymptomatic (strains HCR3A and HCR3B) or symptomatic (strain Ro1845) clinical forms of disease (*4*,*5*,*12*). Strain PA260/97 in the 2-year-old child was associated with enteritis severe enough to require hospitalization. Therefore, the results of this study reinforce the hypothesis that canine-like rotaviruses may be able to not only cross the species barriers but also to induce severe disease forms in children. The lack of systematic surveillance of rotavirus infection in small animals (e.g., dogs and cats) and the fact that most rotavirus infections in such animals

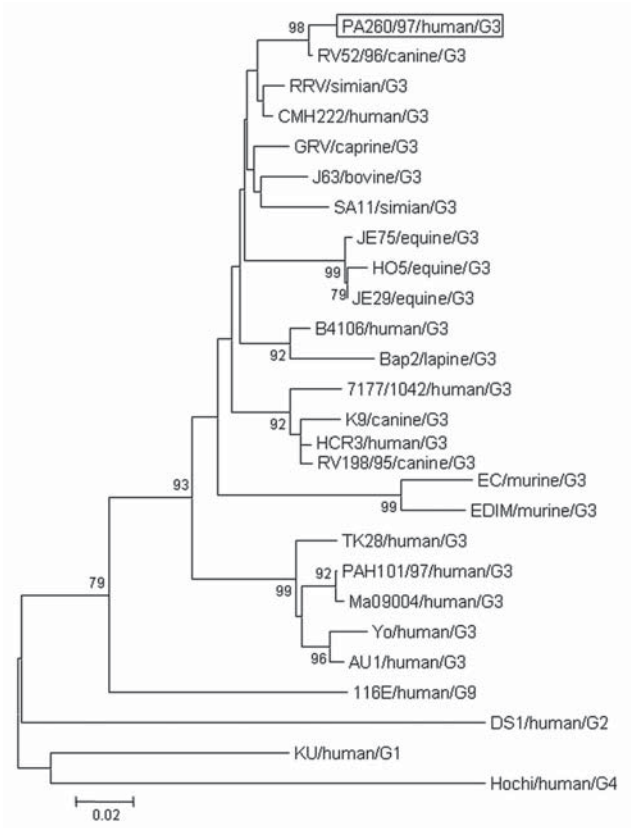

Figure 2. Phylogenetic analysis of deduced amino acid sequence derived from VP7 gene of the PA260/97 human rotavirus strain and other G3 rotavirus genotypes. The tree was generated by the neighbor-joining method using the ClustalW program (http://dambe.bio.uottawa.ca/dambe.asp). Scale bar indicates nucleotide substitutions (×100).

may go undetected hinder the ability to establish firm epidemiologic connections. In conclusion, complementing the human rotavirus surveillance programs with surveillance in animals is paramount to understanding the global ecology of rotaviruses and to identifying and characterizing interspecies transmission events and virus evolution.

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Dr De Grazia is a microbiology specialist and research assistant at the University of Palermo, Dipartimento di Igiene e Microbiologia "G. D'Alessandro." Her primary research interests are microbial typing, viral enteric pathogens, and viral epidemiology.

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Address for correspondence: Serenella Arista, Dipartimento di Igiene e Microbiologia, Università di Palermo, via del Vespro 133, 90127, Palermo, Italy; email: arista@unipa.it

Possible Zoonotic Transmission of Hepatitis E from Pet Pig to Its Owner

Christophe Renou,* Jean-Francois Cadranel,† Marc Bourlière,‡ Philippe Halfon,§ Denis Ouzan,¶ Hervé Rifflet,# Philippe Carenco,* Abdelouahid Harafa,* Jean Jacques Bertrand,* Annie Boutrouille, Pierre Muller,* Jean-Pierre Igual,* Anne Decoppet,†† Marc Eloit,** and Nicole Pavio****

Hepatitis E is transmitted mainly by water or food, but in industrialized countries, all routes of transmission have not been identified. We describe possible zoonotic transmission of hepatitis E virus that involved direct contact between a pet pig and its owner.

Hepatitis E virus (HEV) infections occur sporadically in industrialized countries, where this virus is not endemic, although these infections were initially reported only in persons who had traveled to countries where the virus is endemic. An increasing number of autochthonous cases of HEV have been recently reported in industrialized countries such as Japan, Greece, Spain, Italy, the United States, and France (*1*).

Several lines of evidence suggest that swine, which are also sensitive to HEV infection, may act as a reservoir of the virus and that HEV infections in humans in industrialized countries might be zoonotic. Antibodies to HEV have been detected in domestic pigs in regions where HEV is not endemic (*2*). These antibodies have been detected more frequently in humans occupationally exposed to swine than in those not exposed to swine (*3*). Human and swine HEV strains are closely related genetically (*4*–*7*), and experimental infections of swine with strains from humans and nonhuman primates have been reported (*8*). Moreover, sporadic human cases of acute HEV infection linked with consumption of raw or insufficiently cooked meat (boar or deer) have been reported in Japan (*9*). Therefore, HEV is likely a zoonotic virus that can be directly transmitted by some types of meat or possibly by direct contact with infected animals.

As in other industrialized countries, sporadic autochthonous HEV infections have recently occurred in France (*1,10*). However, the routes of transmission for such infections have not been clearly identified, and this information is needed to characterize the clinical epidemiology of HEV.

The Patient

We report a 41-year-old patient in France with isolated episodes of ≈1-month duration of fatigue since the end of September 2005. Analysis of a serum sample obtained from the patient on day 1 of consultation (October 21, 2005) showed markedly increased liver enzyme levels (aspartate aminotransferase 393 IU/L, alanine aminotranferase 1,211 IU/L), although no associated cholestatic biochemical symptoms were present. The patient lived alone in an urban area, had not traveled abroad for at least 1 year, drank alcoholic beverages only occasionally, and had not recently received any intravenous injections or taken any drugs. Test results were negative for serologic markers of hepatitis A, B, and C, and tests for Epstein-Barr virus and cytomegalovirus detected immunoglobulin G (IgG) for previous infections. Antibodies to HEV were detected in his serum sample by using an enzyme immunoassay (HEV ELISA; Genelabs Diagnostics, St Ingbert, Germany). A positive result (optical density/cutoff value >1) was obtained for HEV-specific IgM (absorbance 8.9); HEV-specific IgG was not detected (optical density/cutoff value ≤1).

HEV RNA was detected in the serum sample by using a nested reverse transcription–PCR for open reading frame (ORF)1 or ORF2 genes (*11*,*12*). The amplified fragments were sequenced directly and aligned with those of other HEV strains. A genotype 3 isolate was identified by using MEGA 3.1 software (www.megasoftware.net). This genotype was similar to those of other European isolates but was specific to this case, which suggested autochthonous local transmission (GenBank accession nos. EF514587 and EF050798) (Figures 1, 2).

Detection of HEV-specific IgM and viral RNA in the patient resulted in a diagnosis of hepatitis E. Gradual clinical and biochemical improvement occurred spontaneously in this patient. Eight weeks before the onset of fatigue, the patient had been given a 3-month-old Vietnamese pig that had been born in France. The pig urinated and defecated outside, and the patient regularly changed the litter. The animal often entered the house and was frequently handled by its owner. In early November 2005, serum samples were collected from the pig and tested for HEV. HEV-specific IgG was not detected, but HEV RNA was detected. As

*Centre Hospitalier d'Hyères, Hyères, France; †Centre Hospitalier, Creil, France; ‡Hôpital Saint-Joseph, Marseille, France; §Hôpital Ambroise Paré, Marseille, France; ¶Institut Arnaud Tzanck, Saint-Laurent du Var, France; #Centre Hospitalier, Ajaccio, Corsica, France; **Agence Française de sécurité Sanitaire des Aliments, Ecole Nationale Vétérinaire d'Alfort, Institut National de la recherche Agronomique, Maisons-Alfort, France; and ††Direction Départementale des Affaires Sanitaires et Sociales du Var, Toulon, France

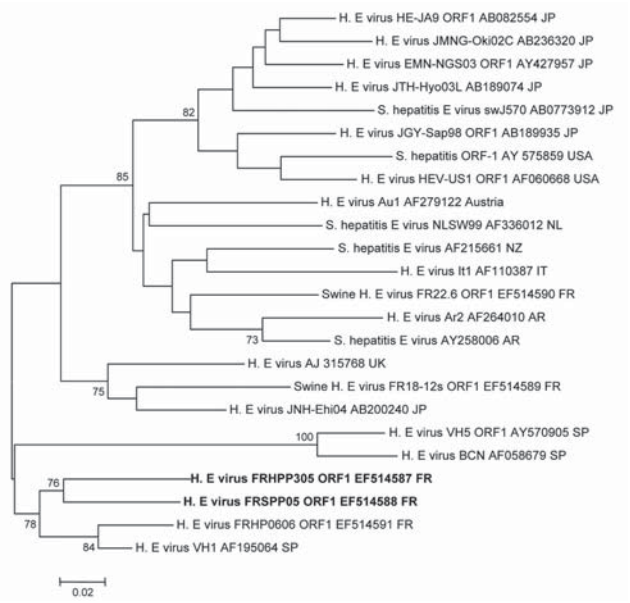

Figure 1. Phylogenetic tree (neighbor-joining method) of hepatitis E virus (HEV) genotype 3 isolates for a 210-nt sequence within the open reading frame (ORF) 1 gene (corresponding to nt 167–376 of the prototype swine genotype 3 pSHEV-3 AY575859). Patient (EF514587) and pet pig (EF514588) sequences are in **boldface** and were compared with French isolates or known isolates from regions where HEV is not endemic. GenBank accession no. and country of origin are indicated. Reliability of the different phylogenetic groupings was evaluated by using a bootstrap test (1,000 replications); scores >70% are indicated. Scale bar indicates no. of nucleotide substitutions per site. JP, Japan; USA, United States; NL, the Netherlands; NZ, New Zealand; IT, Italy; FR, France; AR, Argentina; UK, United Kingdom; SP, Spain.

with the patient, a genotype 3 strain of HEV was identified in serum from the pig by phylogenetic analysis (GenBank accession nos. EF514588 and EF050797) (Figures 1, 2). Isolates obtained from the patient and the pet pig showed homology of 92% at the nucleotide level and 98% at the amino acid level in the ORF2 gene, which has a similar degree of variability as that of the entire HEV genome.

Phylogenetic analyses of HEV genotype 3 sequences for isolates from France and other countries where the virus is not endemic showed a similarity to sequences for the ORF1 and ORF2 genes in isolates from the French patient and his pig. These patient and pet sequences were different from sequences for isolates from patients in the same area of France (EF514591-ORF1, EF050799-ORF2) and from contemporary sequences detected in isolates from French swine herds (EF514589-, EF514590-ORF1; EF494700-, EF494701-, EF494702-, EF494703, EF494704-ORF2) (Figures 1, 2).

Conclusions

The divergence (8%) observed between sequences from the patient and his pet pig is consistent with a variable degree of homology (90%–94%) within 10 HEV subtypes of genotype 3. Furthermore, similar to other RNA viruses, such as hepatitis A and C, HEV is a quasispecies (*13*). In cases of acute hepatitis E contracted by consumption of infected meat, 100% homology between isolates from humans and infected animals was observed, which indicates that during ingestion, all quasispecies are transmitted. In the present case, the route of transmission was different and might have involved selection of variants with a greater zoonotic infection potential by the fecal-oral route. We did not investigate quasispecies variability in this study because of low residual viremia (not detected by single PCR). The unusually prolonged viremia in the pet pig might have resulted in accumulation of mutations over time.

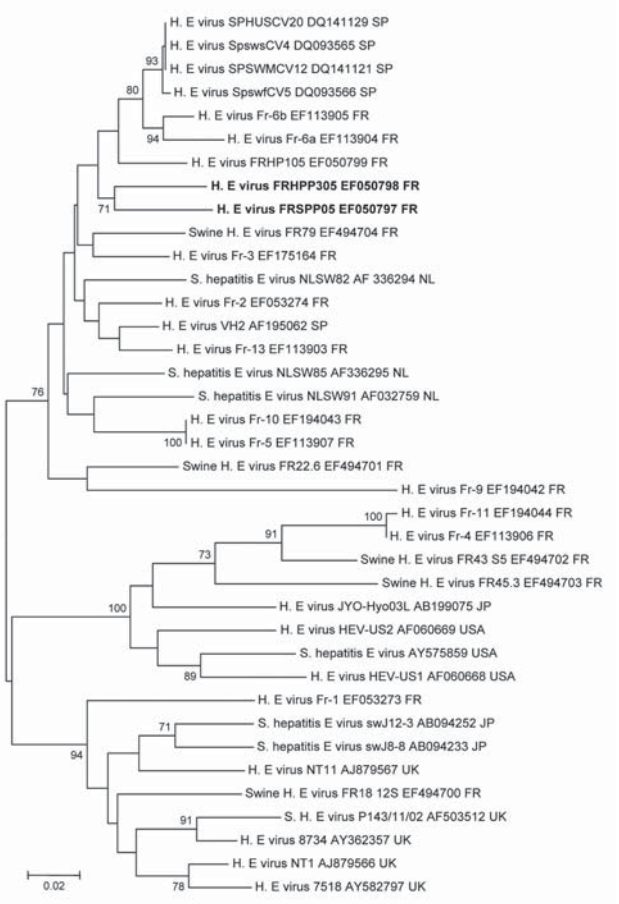

Figure 2. Phylogenetic tree (neighbor-joining method) of hepatitis E virus (HEV) genotype 3 isolates for a 273-nt sequence within the open reading frame 2 gene (corresponding to nt 6078–6350 of the prototype swine genotype 3 pSHEV-3 AY 575859). Patient (EF050798) and pet pig (EF050797) sequences are in **boldface** and were compared with French isolates or known isolates from regions where HEV is not endemic. GenBank accession no. and country of origin are indicated. Reliability of the different phylogenetic grouping was evaluated by using a bootstrap test (1,000 replications); scores >70% are indicated. Scale bar indicates no. of nucleotide substitutions per site. SP, Spain; FR, France; NL, the Netherlands; JP, Japan; USA, United States; UK, United Kingdom.

Evidence suggests that direct zoonotic transmission was involved in this case. The possible incubation time of 8 weeks corresponds to the time between acquisition of the pig and onset of the symptoms. Excretion of HEV by the pet pig probably occurred soon after the patient received it, when the animal was ≈3 months of age. This is consistent with what is observed in natural infections, in which high viral titers are observed at ≈12–15 weeks of age. Although viremia usually lasts ≥3 weeks in a swine experimental model of HEV (*14*), persistence of residual viremia might be explained by the lack of seroconversion observed and the absence of HEV-specific IgG in the pig. Consistent with the genetic variability of HEV and quasispecies, sequences amplified from virus isolates from the patient and the pig were phylogenetically related (ORF1 and ORF2 genes). The difference observed between the 2 sequences suggests that accumulation of mutations occurred during the unusual prolonged viremia observed in the pet pig. There was frequent contact between the pet pig and its owner, including changing the litter, and such contact is presumably responsible for animal-to-human transmission in persons with occupational exposure to swine (*3*). Other possible sources of infection were unlikely. These sources include contaminated water (the pig owner used only the local urban water supply and had no domestic wells) and consumption of undercooked or raw pork (the patient reported that he did not eat pork).

Isolation of virus with related HEV sequences from the patient and his pet pig suggests that the most likely route of transmission was from pig to human. This case therefore supports the current assumption that HEV may be a zoonotic virus and that domestic pet swine are 1 of the natural hosts of HEV. However, the source of HEV infection for such animals is not known and still being investigated.

Dr Renou is a clinician in the department of hepato-gastroenterology at Hyères Hospital in Hyères, France. His research interests include viral hepatitis E and specific routes of transmission in areas not endemic for this disease.

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Address for correspondence: Nicole Pavio, Unité de Mixte de Recherche 1161 Virology, Agence Française de Sécurité Sanitaire des Aliments, Ecole Nationale Vétérinaire d'Alfort, Institut National de la Recherche Agronomique, 7 Ave du General de Gaulle, 94704 Maisons-Alfort CEDEX, France; email: npavio@vet-alfort.fr

Virus Detection and Monitoring of Viral Load in Crimean-Congo Hemorrhagic Fever Virus Patients

Roman Wölfel,*† Janusz T. Paweska,‡ Nadine Petersen,† Antoinette A. Grobbelaar,‡ Patricia A. Leman,‡ Roger Hewson,§ Marie-Claude Georges-Courbot,¶ Anna Papa,# Stephan Günther,† and Christian Drosten†

We developed a real-time reverse transcription–PCR that detected 1,164 copies/mL of Crimean-Congo hemorrhagic fever virus per milliliter of serum at 95% probability (probit analysis) and was 100% concordant with nested PCR on 63 samples from 31 patients with confirmed infection. Infected patients who died appeared to have higher viral loads; low viral loads correlated with IgG detection.

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne viral zoonosis that occurs widely in Africa, Asia, and Eastern Europe. It is caused by *CCHF virus* (CCHFV), a segmented, negative-stranded RNA virus belonging to the family *Bunyaviridae*, genus *Nairovirus*. CCHF has a fatality rate of ≈30% and a potential for nosocomial spread (*1*). Early diagnosis of CCHF is important for case management and protection of medical staff.

Diagnostic assays for CCHF include virus culture, antigen-detection enzyme immunoassay (EIA), antibody-detection EIA, and reverse transcription–PCR (RT-PCR) (*2*). Virus detection is the main diagnostic method in the acute stage of disease, and RT-PCR is most sensitive method of detection. However, because of the remarkable genetic variability among CCHFV strains, all current RT-PCRs either lack sensitivity or focus on the detection of local CCHFV variants only (*3–6*).

We describe the first real-time RT-PCR that rapidly and reliably detects the global spectrum of clinically relevant virus strains. An extended strategy of probe design was implemented to cover such high variability. Sensitivity was demonstrated by testing virus strain collections from several different Biosafety Level 4 laboratories, essentially covering the full range of global diversity of CCHFV (Figure 1). A comprehensive panel of original clinical samples from persons with confirmed cases of CCHF was used for clinical evaluation; the samples were collected by World Health Organization reference facilities.

The Study

Primers and probes were selected on the basis of an alignment of S segment sequences of 61 CCHFV isolates from all known CCHF-endemic regions worldwide (*7*) (representative sequences shown in expanded online version of Figure 2, available from www.cdc.gov/EID/content/13/7/1097-G2.htm). Oligonucleotide melting points, folding characteristics, and cross-hybridization properties were determined by using the Primer Express software package (Applied Biosystems; Foster City, CA, USA). Primers were selected to amplify a 181-bp region near the 5′-end of the S segment. The capability of these primers to amplify 12 representative CCHFV strains from distinct CCHF-endemic regions was confirmed initially by gel detection RT-PCR (data not shown) (Figure 2, expanded online version, panel B).

For real-time PCR, identifying a simple detection probe compatible with all known CCHFV strains was not possible. Therefore, a broad-range probe was formulated

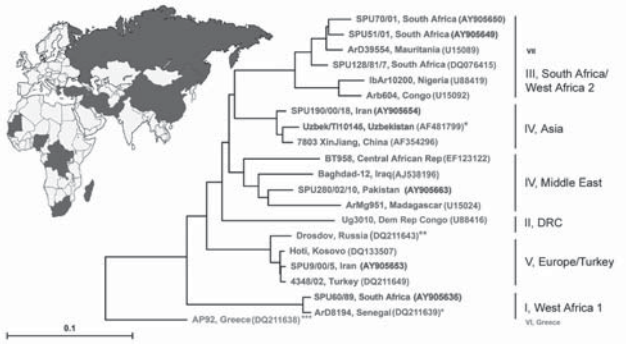

Figure 1. Global distribution and phylogenetic relationships of Crimean-Congo hemorrhagic fever virus (CCHFV) strains selected for design and validation of the assay. All strains except those marked with asterisks were tested. Phylogenetic analysis was based on available 450-bp sequences (from the National Center for Biotechnology Information) of CCHFV small (S-) segment and generated by the neighbor-joining method with TreeCon for Windows (version 1.3b; Yves van de Peer, University Konstanz, Germany). Nomenclature of CCHFV clades is based on (*7*). Note that group VII can be resolved only when analyzing the M-segment, not the S-segment as shown here. *These CCHFV strains are shown for reference, but they were not available for testing. **This strain was not available; however, strain Kosovo, which is almost identical, was tested instead. ***Strain AP92 has also not been available for testing. It was isolated from a *Rhipicephalus bursa* tick and has never been associated with human disease.

*Bundeswehr Institute of Microbiology, Munich, Germany; †Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; ‡National Institute for Communicable Diseases, Sandringham, South Africa; §Health Protection Agency, Porton Down, Salisbury, United Kingdom; ¶Institute Pasteur, Lyon, France; and #Aristotle University of Thessaloniki, Thessaloniki, Greece

on the basis of the observation that the non–Watson/Crick base pair G:T is almost as thermodynamically stable as regular Watson/Crick base pairs, whereas A:C is very unstable (*8*). Thus, the probe was placed on the DNA strand that provided more G:T mismatches than complementary A:C mismatches, and the resulting G:T mismatches were not compensated for. As shown in the left panel of Figure 2B (see expanded online version), this probe, designated SE01, detected all 12 representative strains. Because signal intensity varied according to the strain detected, a strain that provided low signal (BT956, Figure 2B expanded online version, left panel) was chosen for evaluation of sensitivity. Its full S segment RNA was cloned and transcribed in vitro to obtain a quantitative RNA standard (*9*). Cloning, in vitro transcription, purification, and quantification were performed as previously described (*10*). End-point dilution showed that single copies of RNA could be detected despite low overall fluorescence (data not shown). Nevertheless, variation in signal intensity between strains was adjusted by the following 2 modifications. First, an additional oligonucleotide (SE03) was introduced at the same binding site as SE01. This probe had 2 effects: first, a pyrimidine base (IUB-code "Y," 50% C and 50% T) was generated at 2 positions of balanced C/T polymorphisms. Second, a "keto" base (IUB-code "K," 50% G and 50% T) resulted at 1 position of total variability (A, C, G, T). RNA from the 12 representative strains was tested, and those strains that still provided low signal were realigned separately. On the basis of the second alignment, an additional probe was selected at an alternative binding site to prevent interference with probes at the first binding site. It was placed on the minus strand to obtain more G:T mismatches than complementary A:C mismatches (see above). The improvement obtained by the additional probes on the set of representative strains is shown in Figure 2B, middle panel (expanded online version). The final assay protocol is summarized in the Table.

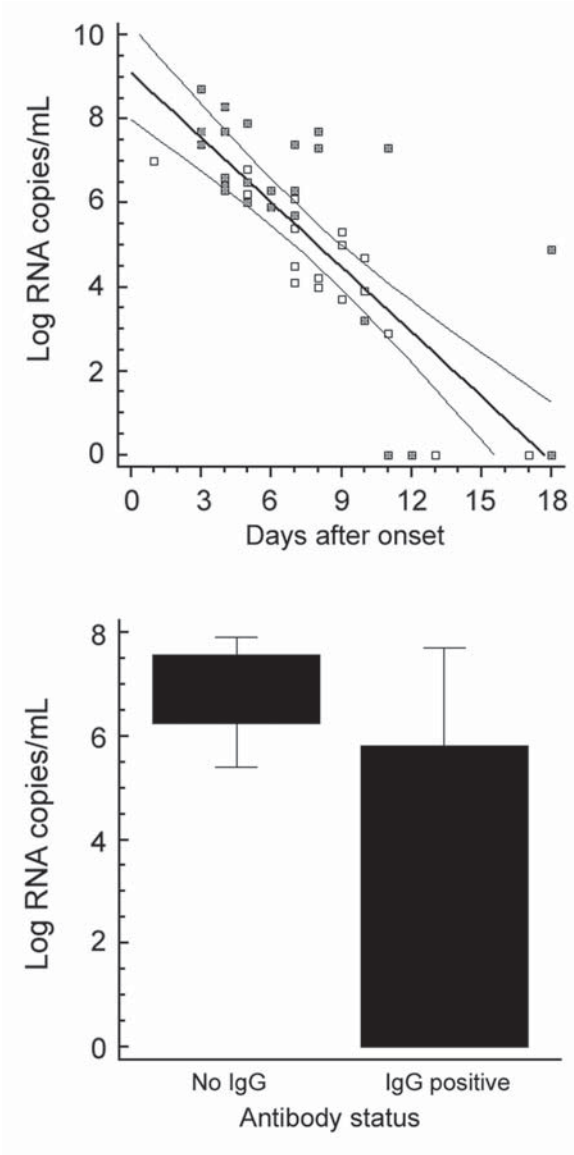

Figure 2. Clinical evaluation. Upper panel: plasma viral load over time in 44 samples from 17 patients. Samples from patients who died are marked with a filled square. Lower panel: plasma viral load in relation to antibody status in 16 samples with and 27 samples without detectable immunoglobulin G (IgG) antibodies. Only IgG status was taken as reference because only 2 patients had IgM without IgG. The difference of means between the 2 groups was highly significant (*t* test, p = 0.00005). Boxes indicate 25th through 75th percentiles; error bars indicate 5th and 95th percentiles.. An expanded online version of this figure is available from www.cdc.gov/EID/content/13/7/1097-G2.htm.

For precise evaluation of analytical sensitivity, a series of human plasma samples was spiked with the RNA standard from strain BT-958 in concentrations ranging from 100,000 to 10 copies per mL. Testing was done on 5 replicate reactions per concentration, and probit analysis was conducted as shown in the expanded online version of Figure 2, panel B, right graph (*11*). The calculated limit of detection, defined as the concentration down to which >95% of conducted tests can be expected to be positive, was 13.6 copies per reaction (p = 0.05). This corresponded to 1,164 copies per mL of plasma (95% confidence interval, 780–2990 copies/mL).

Cross-reactivity was excluded by testing DNA or RNA from cultures or high-titered clinical samples containing Dugbe virus, Rift Valley fever virus, Sudan Ebolavirus Gulu, Lassa virus AV, yellow fever virus, dengue virus types 1–4, Japanese encephalitis virus, West Nile virus Uganda, Venezuelan equine encephalitis virus, Sindbis virus, Ross River virus, Epstein-Barr virus, hepatitis C virus, human cytomegalovirus, monkeypox virus, poliomyelitis virus types 1–3, rabies virus RSDD, *Bacillus anthracis*, *Leptospira interrogans*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Coxiella burnetii, Rickettsia prowazekii, R. rickettsii,* and *Plasmodium falciparum*. An additional

Table. Protocol for real-time reverse transcription–PCR

Oligonucleotide*	Purpose, concentration in nM	Sequence and label (5′→3′)	Position (U88410)†
RWCF	Forward primer, 600	CAAGGGGTACCAAGAAAATGAAGAAGGC	1068–1095
RWCR	Reverse primer, 600	GCCACAGGGATTGTTCCAAAGCAGAC	1248–1223
SE01	Broad-range probe, 100	FAM-ATCTACATGCACCCTGCTGTGTTGACA-TAMRA	1172–1198
SE03	Additional probe, 100	FAM-ATTTACATGCACCCTGCCGTGCTTACA-TAMRA	1172–1198
SE0A	Additional probe, 100	FAM-AGCTTCTTCCCCCACTTCATTGGAGT -TAMRA	1131–1106

*All oligonucleotides were used in an assay with the following protocol: 25-µL reaction volume, 5-µL plasma RNA (QIAamp Viral RNA mini kit; QIAGEN, Valencia, CA, USA), 1× concentration of buffer and enzymes from the OneStep RT-PCR kit (QIAGEN), and 400 µmol dNTP, 800-ng nonacetylated bovine serum albumin (Sigma-Aldrich, Munich, Germany). The cycling parameters followed in a Roche LightCycler 1.2 (Roche, Penzberg, Germany) were as follows: 30 min at 50°C, 15 min at 95°C, 46× 15 s at 94°C and 30 s at 59°C. Fluorescence acquisition occurred at the 59°C step, wavelength filter F1/F2 mode.
†GenBank accession no.

128 blood specimens collected during the course of the study from 128 patients with conditions other than CCHF all tested negative for CCHF virus.

The real-time RT-PCR was used to test and quantify 63 serum samples from 31 patients with laboratory-confirmed CCHFV infection; the samples were obtained 1–18 days after symptom onset. All samples had nested RT-PCR results positive for CCHFV (*3*), and all were also positive by the new real-time RT-PCR. For 21 patients with confirmed CCHF (17 from South Africa, 3 from Iran, and 1 from Pakistan), viral load was quantified and compared with other standard diagnostic methods for CCHFV detection (online Appendix Table, available from www.cdc.gov/EID/content/13/7/1097-appT.htm). Again, sensitivity of the new assay was at least as high as that of nested PCR. As shown in Figure 2, there was a clear correlation between viral load and duration of symptoms in these patients. Clinical outcome could not be correlated clearly with viral load, although patients who died of the disease seemed, in general, to have higher viral loads (Figure 2, filled squares). The appearance of antibodies correlated clearly with lower viral loads (Figure 2).

Conclusions

To our knowledge, this is the first PCR validated with representative CCHFV strains from nearly all regions worldwide where the virus is endemic. High sensitivity enables reliable detection of virus in early stages of the infection, when antibody detection is unreliable or impossible. By eliminating the need for postamplification product processing, real-time RT-PCR enables shortened turnaround times for reporting results, which is critical for deciding on isolation and contact-tracing for suspected case-patients. Quantification of viral load may assist in estimating the patient's infectivity. It may also assist in predicting the clinical outcome and could be used to monitor viral load in patients receiving ribavirin treatment (*12*). Our study provides baseline data on CCHF viral load throughout the acute stage of the illness. High viral load tended to indicate fatal outcome, and lower viral load was generally associated with detectable antibodies. Because detectable antibody response correlates with good outcome (*13*), viral load will probably be a useful predictor of clinical progress. These preliminary data are highly encouraging for further studies on larger patient cohorts.

Acknowledgments

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Dr Wölfel is a medical microbiologist at the Department of Virology and Rickettsiology of the Bundeswehr Institute of Microbiology in Munich, Germany. He is working in the area of medical defense against biological warfare and terrorism, and his interests include viral hemorrhagic fevers and rickettsial diseases.

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Address for correspondence: Christian Drosten, Bernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Str 74, 20359 Hamburg, Germany; email: drosten@bni-hamburg.de

Zinc Cream and Reliability of Tuberculosis Skin Testing

V. Bhargavi Rao,*† Tom F. Pelly,*‡ Robert H. Gilman,‡§¶ Lilia Cabrera,‡ Jose Delgado‡ Giselle Soto,‡ Jon S. Friedland,* A. Roderick Escombe,*‡ Robert E. Black,¶ and Carlton A. Evans*†‡§¶

In 50 healthy Peruvian shantytown residents, zinc cream applied to tuberculosis skin-test sites caused a 32% increase in induration compared with placebo cream. Persons with lower plasma zinc had smaller skin-test reactions and greater augmentation with zinc cream. Zinc deficiency caused false-negative skin-test results, and topical zinc supplementation augmented antimycobacterial immune responses enough to improve diagnosis.

Tuberculosis (TB) kills >1.7 million people each year, and control is hampered by diagnostic difficulties. The TB (Mantoux) skin test measures the immune response to an intradermal injection of tuberculin (purified protein derivative [PPD]) and is important for diagnosing adult and particularly pediatric TB (*1*). However, reliability of this skin test is limited by false-negative results (*2*–*4*), especially in poorly nourished people in the resource-limited settings where most cases of TB occur (*2*). Zinc is implicated in false-negative skin tests because it modulates cutaneous reactions (*2*,*3*), because zinc deficiency is common in people with TB (*5*), and because this deficiency also suppresses antimycobacterial immunity (*4*,*5*). We therefore studied whether topical application of zinc to TB skin test sites would augment test results in Lima, Peru, a TB-endemic area in which false-negative skin-test results are frequent (*1*).

The Study

After ethical approval and informed written consent were obtained, venous blood was collected from 50 healthy, randomly selected, adult shantytown resident volunteers. Plasma zinc concentrations were analyzed blindly by atomic absorption spectroscopy; precautions were taken against trace-metal contamination. In each study participant, the volar surface of both forearms was injected proximally with tuberculin (5 U in 0.1 mL; Aventis-Pasteur, Toronto, Canada) and distally with *Candida albicans* antigen (5 U; Hollister Stier Laboratories, Spokane, WA, USA) (*1*), totaling 4 simultaneous skin tests per person. *Candida* skin tests were conducted because, when positive, they confirm that the person can mount a cutaneous hypersensitivity reaction, which clarifies that a simultaneous negative TB skin-test result is likely to be a true negative.

In a randomized double-blind manner, skin-test sites on 1 arm were covered with 1 mL of placebo cream (Aqueous cream BP, Sandoz, Bordon, Hants, UK) and the skin-test sites on the contralateral arm were covered with zinc sulfate (*2*) dissolved in aqueous cream to a concentration of 1% elemental zinc. Each skin test site was immediately covered with an occlusive dressing (Tegaderm, 3M, London, UK). After 24, 48, and 72 h, the dressing and cream were removed, and the "ball-point-pen" test was used to measure induration. The appropriate cream was reapplied and covered after each measurement.

SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Except where otherwise stated, the results of skin tests are those read after 48 h. Continuous data were all normally distributed (except for food frequencies) and were therefore summarized as means (and standard errors of the mean [SEM]). Groups were compared by using *t* tests, and associations were tested by using univariate linear regression (p value and standardized coefficient shown) and in a backward multiple linear regression model with the least significant variables sequentially removed, according to the log-likelihood test.

Conclusions

Control TB skin-test areas to which placebo cream had been applied yielded significantly smaller reactions in persons with lower concentrations of plasma zinc ($p = 0.03$, Table). For example, the quarter of the population with the lowest concentrations had, on average, control TB skin-test reactions that were 14 mm in diameter compared with 27 mm for the quarter of the population with the highest plasma zinc concentrations ($p = 0.03$). This immunologically significant zinc deficiency was frequent even though only 10% of the study population was underweight (body mass index <20 m^2/kg).

The TB skin-test results were read in a blinded manner, and reactions to tests with zinc cream applications were an average of 32% larger than the contralateral control skin test reactions with placebo cream applied (Figure 1; $p<0.001$). TB skin-test results were considered positive if the average diameter of induration was >10 mm 48 h after injection, according to national policy. Skin tests with zinc applications were significantly more likely to have positive results

*Imperial College London, London, UK; †London School of Hygiene and Tropical Medicine, London, UK; ‡Asociación Benéfica Prisma, Lima, Peru; §Universidad Peruana Cayetano Heredia, Lima, Peru; and ¶Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA

Table. Population characteristics for the participants who had simultaneous skin tests with and without zinc and associations with size of tuberculosis (TB) skin-test reaction

Characteristic*	Mean (SEM)† or % (n = 50)	Association with size of control TB skin-test reaction (mm)			
		Univariate analysis		Multiple regression	
		Coefficient	p	Coefficient	p
Nutritional assessment‡§					
Body mass index (kg/m^2)	24 (0.52) 10% underweight (<20)	0.2	0.3		
Anthropometric protein status (corrected arm muscle area; cm^2)	36 (1.4)	0.2	0.09		
Anthropometric fat status (arm fat area; cm^2)	14 (1.1)	0.0008	1		
Plasma zinc (mg/L; n = 49)	0.66 (0.17) 31% deficient (≤0.6)	0.3	0.04	0.3	0.03
Minor incidental health symptoms	38%	0.06	0.7		
TB risk factors					
Age, y	32 (1.3)	0.2	0.2	0.3	0.02
Male	38%	0.3	0.03	0.4	0.01
Past close contact with a TB patient or past proven diagnosis of TB	42%	0.1	0.4		
Presence of Baccilus Calmette-Guérin vaccine scar(s)	86%	0.2	0.3		
Overcrowding (persons/room)	1.3 (0.08)	–0.2	0.9	0.2	0.09
Poor household ventilation (subjective assessment)	24%	–0.002	1		
Socioeconomic status					
Food spending/person/d ($US)	0.75 (0.05)	0.1	0.5		
Dirt floor throughout the home	38%	–0.06	0.7		
Home built from temporary materials	72%	0.1	0.5		
No in-house sanitation	84%	0.05	0.7		
No piped water to home	84%	–0.07	0.6		

*Variables that may influence TB skin-test reaction size are shown together with their associations with the size of the control TB skin-test reactions associated with only zinc cream application.
†SEM, standard error of the mean.
‡Food frequencies: in the previous week, alcohol had been consumed a median of 1 time (median 1 U), dairy produce 3 times, fruit/vegetables 5 times, meat/fish 6 times, and rice/bread/cereals daily.
§Persons with infrequent meat/fish consumption had lower protein stores on muscle anthropometry (p = 0.02), and this tended to be associated with smaller skin-test reactions, as previously reported (*1,2*).

than the simultaneous contralateral control skin tests with placebo cream applications (94% zinc vs. 76% placebo, p = 0.01). Topical zinc caused greater TB skin-test augmentation in persons with lower plasma zinc concentrations (correlation coefficient [*R*] = 0.3, p = 0.05), and persons with absolute plasma zinc deficiency (<0.6 mg/L) had significantly greater zinc-mediated augmentation (Figure 2, p = 0.02). Zinc cream had no effect in persons with normal plasma zinc concentrations. Thus, zinc cream significantly augmented TB skin-test reactions only in persons deficient in zinc (Figure 2).

TB skin tests are usually read 48 h after PPD injection; however, in this study, additional readings were done at 24 and 72 h, at which time results, similar to those at 48 h, demonstrated that zinc cream caused larger TB skin-test reactions (both p<0.02) and more positive test results (both p<0.006). Furthermore, at 72 and 48 h, this zinc-mediated augmentation was greater in zinc-deficient persons (p = 0.006), although the similar trend at 24 h was not statistically significant. The magnitude of skin-test augmentation with zinc cream, the plasma zinc concentration, and the anthropometric measures had no significant associations with other studied factors except as noted in the Table.

The effect of zinc on the *Candida* skin-test results was similar to the effect on the TB skin-test results. Specifically, the *Candida* skin-test reactions with zinc cream were an average of 23% larger than the contralateral skin-test reactions associated with placebo cream applied in a double-blind manner (p<0.001). *Candida* skin-test results were considered positive if the average diameter of induration was >5 mm (*1*), and 78% of *Candida* skin-test results associated with zinc application were positive, compared with 64% with placebo cream (p = 0.01). Zinc-mediated TB and *Candida* skin-test augmentation were associated (*R* = 0.3, p = 0.05), but zinc-mediated *Candida* skin-test augmentation was not significantly associated with any of the variables in the Table.

The control skin tests with placebo cream yielded larger reactions (p = 0.005) and were more likely to be positive (p = 0.005) than concurrent skin tests on 97 other randomly

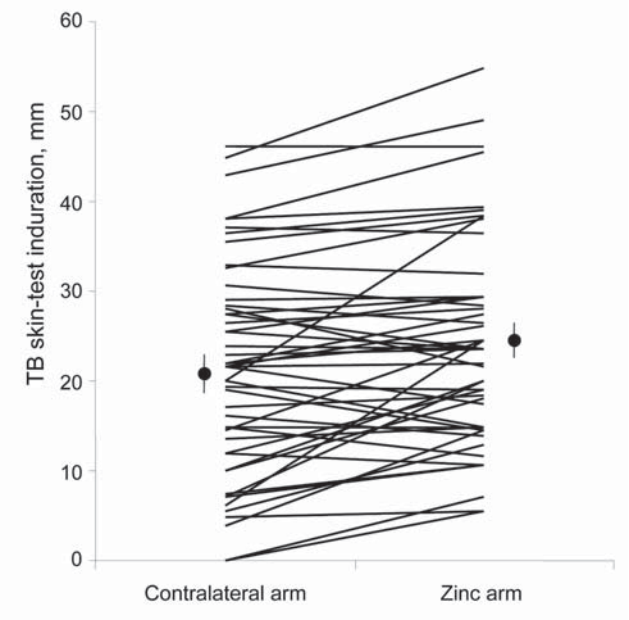

Figure 1. Effect of topical zinc on size of tuberculosis (TB) skin-test reaction. Each line represents the size of the 2 TB skin-test reactions for 1 person with placebo cream (on the left of the graph) and topical zinc cream (on the right). Circles show the mean TB skin-test reaction size without and with topical zinc, with standard error bars.

selected healthy volunteers in the same community (data not shown). These 97 skin tests were each performed on 1 arm only, and no creams were applied; the mean diameter of induration was 16.4 mm (SEM 1.9 mm), and 52% were TB skin-test positive (>10 mm). Although the design of this part of the study prevented blinded assessment, this comparison implies that in the 50 persons with simultaneous bilateral skin tests, zinc absorption through the skin may have partially augmented the contralateral control skin-test reactions to which placebo cream was applied. Although the local direct effect at the site of zinc cream application was significantly greater, systemic zinc absorption may have caused the effect of zinc cream to be underestimated; this hypothesis is being further investigated.

Zinc status is difficult to reliably assess (*2*–*4*). However, low plasma zinc significantly predicted small skin-test reactions and the magnitude of the immunologic response to topical supplementation. Thus, zinc cream had a specific effect, namely, reversing the skin-test suppression of zinc deficiency. Topical zinc supplementation applied to the arms of persons who received TB skin tests therefore identified persons in whom zinc deficiency was sufficient to suppress antimycobacterial immune responses and quantified the immune-potentiating effect of supplementation. This approach may similarly facilitate the therapeutic evaluation of other micronutrients and immunomodulatory compounds.

Zinc cream had no effect in persons with adequate concentrations of zinc; however, persons who were deficient in zinc but otherwise apparently healthy had suppressed skin-test reactions, despite the absence of frank malnutrition; this suppression was reversed by the application of zinc cream. Thus, zinc cream application corrected false-negative TB skin-test reactions caused by zinc deficiency, allowed more sensitive diagnosis, and hence facilitated appropriate treatment of latent TB infection. Topical zinc is a simple and relatively inexpensive method of enhancing reliability of the established TB skin test for diagnosing this neglected disease. This has public health implications because the TB skin test is central to the problematic area of diagnosing TB infection and disease, especially in children who are prone to both zinc deficiency (*2*,*4*) and false-negative skin-test results (*1*). Therefore, the zinc-mediated augmentation of TB skin testing that we have demonstrated may facilitate the diagnosis of adult and pediatric cases of TB in regions where micronutrient deficiency is prevalent.

In conclusion, this study demonstrated that physiologically significant subclinical zinc deficiency was common in this population, that low plasma zinc predicted negative TB skin-test results, and that topical zinc supplementation augmented local antimycobacterial immune responses sufficiently to reverse this anergy of micronutrient deficiency.

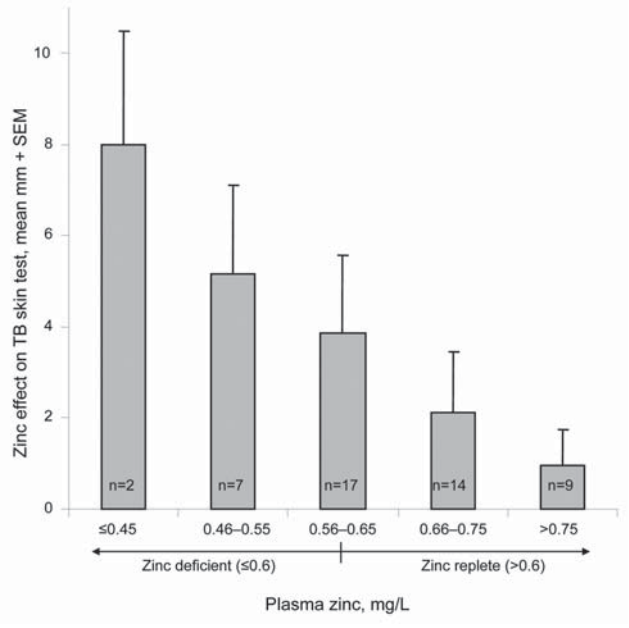

Figure 2. Association between plasma zinc concentration and response to topical zinc. The association is shown between plasma zinc concentration and the magnitude of augmentation of the purified protein derivative skin test with topical zinc. The normal range of plasma zinc (>0.6 mg/L) is also indicated.

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Dr Rao is a physician specializing in international public health practice and research.

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Address for correspondence: Carlton A. Evans, Asociacion Benefica Prisma, Av Carlos Gonzales Nro. 251, Urb. Maranga, San Miguel, Lima 32, Peru; email: carltonevans@yahoo.com

Three Rickettsioses, Darnley Island, Australia

Nathan B. Unsworth,* John Stenos,* Antony G. Faa,† and Stephen R. Graves*

We report 3 rickettsioses on Darnley Island, Australia, in the Torres Strait. In addition to previously described cases of Flinders Island spotted fever (*Rickettsia honei* strain "marmionii"), we describe 1 case of Queensland tick typhus (*R. australis*) and 2 cases of scrub typhus caused by a unique strain (*Orientia tsutsugamushi*).

The Torres Strait islands are scattered between Cape York in northeastern Australia and Papua New Guinea. Darnley (Erub) Island is the largest island (5.7 km^2) in the eastern Torres Strait and has a mostly indigenous population of 360.

Rickettsioses in northeastern Australia include Queensland tick typhus (*Rickettsia australis*) (*1*,*2*), murine typhus (*R. typhi*), scrub typhus (*Orientia tsutsugamushi*) (*3*), and Flinders Island spotted fever (*R. honei* strain "marmionii") (*4*). The latter 2 diseases are endemic to the Torres Strait islands (*4*,*5*). Because all 4 diseases have similar clinical manifestations, which may include maculopapular rash, fever, headache, rigor, myalgia, and arthralgia (*1–4,*), laboratory investigation is needed to identify the rickettsial etiologic agent. We describe the northernmost case of Queensland tick typhus and 2 cases of scrub typhus, along with their molecular identifications, from patients examined at the Darnley Island Health Clinic.

The Cases

In March 2003, a 23-year-old man (patient 1) sought treatment for fever (39.3°C), headache, and an eschar on his right thigh. He had no rash. No diagnosis was made, but he was given penicillin V. Seven days later he returned with worsening symptoms of fever (39.9°C), headache, cough, arthralgia, and lethargy. A provisional diagnosis of scrub typhus was made, and he was given doxycycline. By the next day he was afebrile, and his condition was much improved. The diagnosis was confirmed by a positive serologic result for scrub typhus rickettsiae (antibody titer 512) and the presence of *O. tsutsugamushi* in blood detected on day 7 by PCR and culture (Table).

In May 2004, an 11-year-old boy (patient 2) was examined because of a 3-day history of fever (39.0°C), headache, nausea, abdominal pain, and a boil surrounded by cellulitis behind his left ear. He had no rash. He was given flucloxacillin for the boil. On day 10 he was still ill and febrile. His blood was tested for malaria, dengue, and scrub typhus, but he was given no antimicrobial therapy. He was not seen again until day 18, when he was prescribed doxycycline after PCR detected *O. tsutsugamushi* (and it was later isolated) in his day-10 blood sample. Follow-up serologic testing of samples collected on days 10 and 33 displayed seroconversion to antibodies against scrub typhus (titers <128 to >1,024; Table).

In April 2003, a 29-year-old man (patient 3) sought treatment for fever (38.4°C), cough, nausea, lethargy, and cellulitis of both feet. His left thigh had 2 boils, and his inguinal nodes were palpable. Three days later, he was evacuated by air and admitted to the Thursday Island hospital, at which time he was still febrile (39.2°C) but had no cellulitis. He also had a macular rash, which he reported as originally having been pustular; the thigh lesions were recognized as eschars. A tentative diagnosis of scrub typhus was made, and the patient was given doxycycline. Within 48 hours he was afebrile and discharged. Serologic testing for rickettsiae was positive for the spotted fever group (titer 256) on day 3, but the titers remained unchanged 4 months later. A spotted fever group rickettsial organism was grown from the day-3 blood sample; however, PCR was negative for rickettsiae (Table).

Rickettsial serologic testing was performed on patients' paired serum specimens by using an indirect immunofluorescence assay (*6*). Titers ≥128 were deemed positive. Patient 1 had an increase in titer to *O. tsutsugamushi;* patient 2 exhibited seroconversion to *O. tsutsugamushi* antibodies; and patient 3 had stationary positive titers to spotted fever group rickettsiae (Table).

Rickettsial isolation was performed according to previously described methods (*7*). A spotted fever group rickettsial organism was isolated from patient 3, and an *Orientia* organism was isolated from patients 1 and 2 (Table). Only 1 *Orientia* organism could be adapted to continuous culture (patient 2).

We extracted DNA from enriched buffy coat and rickettsial cultures by using the QIAamp Blood Mini Kit (QIAGEN, Hilden, Germany) and following the manufacturer's protocols. Scrub typhus was diagnosed by 56-kDa gene PCR, which used the primers A (5′-TACATTAGCTGCAGGTATGACA-3′) and B (5′-CCAGCATAATTCTTTAACCAAG-3′) (Invitrogen, Mount Waverley, Victoria, Australia) as previously described, without the nested procedure and with a 51°C annealing temperature (*8*). Buffy

*The Australian Rickettsial Reference Laboratory, Geelong, Victoria, Australia; and †Warwick Hospital, Southern Downs Health Services District, Warwick, Queensland, Australia

Table. Rickettsial detection, Darnley Island, Australia, 2003–2004

Patient	Serum sample no. 1			Serum sample no. 2			*Orientia tsutsugamushi*		*Rickettsia australis*	
	Days after disease onset	SFG titer*	STG titer†	Days after disease onset	SFG titer*	STG titer†	PCR	Culture	PCR	Culture
1	7	128	512	170	128	>1,024	+	+	–	–
2	10	128	<128	33	128	>1,024	+	+	–	–
3	3	256	<128	133	256	<128	–	–	–	+

*SFG, spotted fever group; antigens were *R. honei*, *R. australis*, *R. akari*, *R. conorii*, *R. siberica,* and *R. rickettsii.*
†STG, scrub typus group; antigens were the *O. tsutsugamushi* strains Gilliam and Kato.

coats and cultures from patients 1 and 2 were PCR positive for *O. tsutsugamushi* (Table). The 320-bp product (patient 2) was sequenced (Newcastle DNA, University of Newcastle, Australia; GenBank accession no. AY860955) and shared 89.8% homology with the Taiwanese strains TW381 and TW521 (GenBank accession nos. AY222635 and AY222630, respectively). A phylogenetic tree of the 56-kDa antigen gene was constructed by using the SEQBOOT and CONSENSE programs of the PHYLIP software package (Figure).

Spotted fever group rickettsemia was identified by 17-kDa antigen gene PCR that used the primers MTO-1 (5′-GCTCTTGCAACTCTATGTT-3′) and MTO-2 (5′-CATTGTTCGTCAGGTTGGCG-3′) (Invitrogen) as previously described, with an annealing temperature of 51°C and 45 cycles (*9*). The 17-kDa buffy coat PCR result was negative for patient 3, but the culture gave a 413-bp sequence that was 100% homologous with *R. australis* (GenBank accession no. M74042; Table). The patient's buffy coat DNA extract was not tested for PCR inhibitors, and no attempt was made to use the *R. australis* isolate in a heterologous serologic reaction because the strain could not be established in continuous culture.

Conclusions

Isolation of *R. australis* from a patient on Darnley Island redefines the northern limit of distribution of Queensland tick typhus in Australia. Previously, Queensland tick typhus had been thought to extend from Wilson's Promontory (the tip of southeastern Australia) (*10*) to the Atherton Tableland (north Queensland) (*2*). This more northern finding of Queensland tick typhus was not unexpected because distribution of the vector of Queensland tick typhus in northeastern Australia, *Ixodes holocyclus*, is likely to include the Torres Strait islands and Papua New Guinea (*11*).

Rickettsial diseases that differ clinically from scrub typhus have been reported in Papua New Guinea (*12*). A recent serologic survey found 7 (3.7%) of 191 Papua New Guineans were seropositive to scrub typhus or spotted fever group rickettsiae (*13*). Another survey found that at least 19 (17%) of 113 Papua New Guineans had an antibody titer >256 against spotted fever group rickettsiae (A.G. Faa, unpub. data). A spotted fever group rickettsial disease such as Queensland tick typhus, Flinders Island spotted fever, or another undescribed rickettsiosis could explain these findings.

Low antibody titers (128) to the spotted fever group in the scrub typhus patients (Table) are consistent with previous exposure to spotted fever group or typhus group rickettsiae. Because 20% of each enriched buffy coat specimen was examined by rickettsial PCR and 80% by culture, results were skewed in favor of isolation rather than DNA detection.

Since 1935, numerous cases of scrub typhus have been reported in Australia and Papua New Guinea (*5*,*12*). Scrub typhus is known to be endemic to Darnley Island; however, strains have not been typed (*5*). Strains from northeastern Australia were serologically determined to be Karp or Karp-related (*14*). However, this new strain is 10.2% divergent from any other described strain, including Karp (Figure). Hence, we designated it as the Darnley strain, after the island from which it was isolated. The

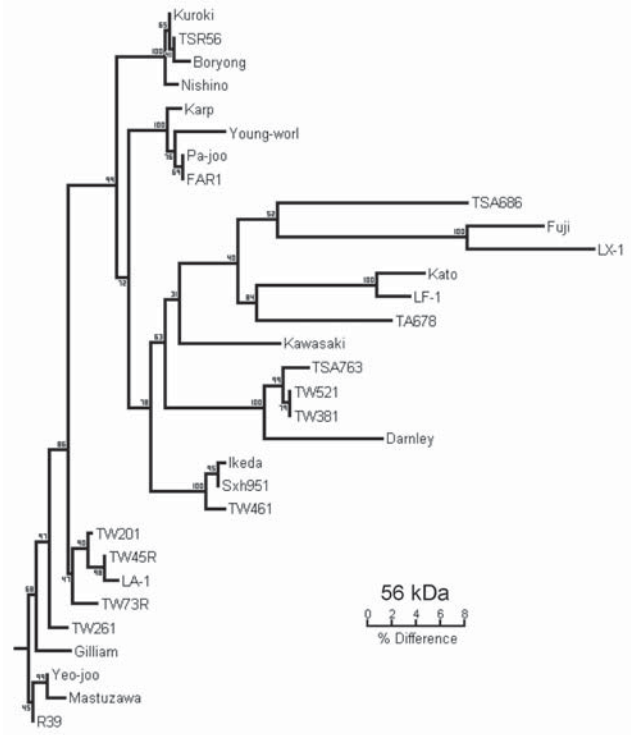

Figure. Phylogenetic tree obtained by a neighbor-joining analysis of the 56-kDa gene of *Orientia tsutsugamushi.* Bootstrap values from 100 analyses are shown at the node of each branch.

phylogenetic relationship of the Darnley strain to other Australian strains, including Litchfield, needs to be elucidated. The presence of 3 rickettsial diseases on this small island demonstrates the complexity of rickettsial epidemiology in Australia.

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Dr Unsworth is a postdoctoral research associate at Texas A&M University, College Station, Texas, USA. His interests include the epidemiology of Australian rickettsiae and Q fever pathogenesis.

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Address for correspondence: John Stenos, The Australian Rickettsial Reference Laboratory, The Geelong Hospital, PO Box 281, Geelong, Victoria, Australia 3220; email: johns@barwonhealth.org.au

Chlamydophila psittaci Transmission from Pet Birds to Humans

Daisy Vanrompay,* Taher Harkinezhad,* Marijke van de Walle,* Delphine Beeckman,* Caroline van Droogenbroeck,* Kristel Verminnen,* Ruud Leten,† An Martel,† and Katty Cauwerts‡

We studied zoonotic transmission of *Chlamydophila psittaci* in 39 breeding facilities for Psittaciformes (cockatoos, parrots, parakeets, lories) that frequently used antimicrobial drugs. Genotypes A or E/B were detected in 14.9% of humans at these facilities. Information on antimicrobial drug use in Psittaciformes and a *C. psittaci* vaccine are urgently required.

Chlamydophila psittaci, an obligate, intracellular, gram-negative bacterium, has 7 known genotypes (A–F and E/B) (*1*), All genotypes can be transmitted to humans and cause psittacosis or parrot fever (*2*). Genotypes are distinguished by sequencing of the outer membrane protein A (*ompA*) gene (*3*) or by a recently developed *ompA* genotype-specific real-time PCR (*4*). *C. psittaci* can infect 465 avian species in 30 avian orders, with at least 153 species in the order Psittaciformes (*5*). From 1988 through 2003, a total of 935 human cases of psittacosis were reported to the US Centers for Disease Control and Prevention (*6*); most were related to contact with Psittaciformes. Currently, ≈100 psittacosis cases are reported annually in the United States, and 1 person may die of this disease each year. The incidence of psittacosis in men seems to be increasing in industrialized countries and is related to importation of exotic birds. Other cases may not be correctly diagnosed or reported.

The Study

We investigated zoonotic transmission of *C. psittaci* in Belgian breeding facilities for Psittaciformes (cockatoos, parrots, parakeets, lories). Participants were recruited through the Belgian Society for Parakeet and Parrot Breeders. Fifty breeding facilities received a sampling package by regular mail. The package contained a questionnaire designed to assess information on the pet bird owners' professional and nonprofessional activities, smoking habits, general health status, use of medication, allergies, clinical signs specifically related to psittacosis; on the birds' origin, housing, feeding, breeding, health status, and medication; and on the presence of other bird species. The package also contained dacron-tipped swabs and instructions for fecal (cage floor) or pharyngeal sampling in birds or humans, respectively. We provided transport medium (*7*) for isolation and DNA stabilization buffer (Roche, Brussels, Belgium) for PCR, to be added to swabs after sampling.

Packages returned by express mail 1 day after sampling were stored at –80°C until use. Sampling packages are convenient and safe for investigators. Forty-one (82%) of 50 breeding facilities returned the packages. Two packages were incomplete and therefore excluded, resulting in samples from 308 birds and 46 humans from 39 Psittaciforme breeding facilities for testing. We also obtained pharyngeal samples from a veterinary student who was involved in another study at the same breeding facilities. This student was examined before our study and every month for 4 months. All humans were examined and provided informed consent.

The *ompA* gene was detected in birds and humans by using a *C. psittaci*–specific nested PCR/enzyme immunoassay (EIA) (*8*). Viable *C. psittaci* were detected by isolation in buffalo green monkey cells and direct immunofluorescence staining (IMAGEN; Dakocytomation, Copenhagen, Denmark) (*7*). When zoonotic transmission occurred, the infection source was traced by using a genotype-specific real-time PCR (*4*) for specimens from birds and their owners.

Fifty-nine (19.2%) of 308 Psittaciformes were positive for *C. psittaci* in the nested PCR/EIA, and bacteria were isolated from 25 (42.3%) birds with PCR-positive results. Of 39 tested breeding facilities, 8 (20.5%) were positive in both the nested PCR/EIA and culture, and respiratory disease was present at all facilities. Five other breeding facilities showed only positive results for the nested PCR/EIA. One of these facilities was currently treating birds with doxycycline, and the remaining 4 had recently used doxycycline, oxytetracycline, or enrofloxacin. Treatment was successful because viable *C. psittaci* were not detected at these 5 breeding facilities, and all their birds appeared healthy. A total of 13 (33.3%) of 39 *C. psittaci*–positive breeding facilities showed a significant correlation between fecal excretion of viable chlamydia and respiratory disease (odds ratio 14.5, 95% confidence interval 1.6–130.5, $p<0.05$).

The remaining 26 breeding facilities with healthy birds had negative results for PCR and culture. Early infections may have been missed because fecal excretion occurs after primary bacterial replication in the respiratory tract and

*Ghent University, Ghent, Belgium; †Ghent University, Merelbeke, Belgium; and ‡Federal Department of Health, Food Chain Safety and Environment, Brussels, Belgium

septicemia. Moreover, fecal shedding occurs intermittently and healthy carrier birds might not excrete bacteria for >1 year. Pharyngeal swabs would have been a better choice for testing but psittacine owners are reluctant to catch their birds because stress induces respiratory disease. Reactivation of a *C. psittaci* carrier status might be involved in this phenomenon.

Nested PCR/EIA detected *C. psittaci* DNA in 6 (13%) of 46 pet bird owners. All 6 owners obtained birds at different breeding facilities, and all facilities had birds positive for *C. psittaci* by PCR and culture. The infected persons were 22, 24, 31, 38, 49, and 56 years of age (mean age of the pet bird owners was 46 years). Viable organisms were present in 4 of 6 persons with positive PCR results, and all 4 (who were nonsmokers and had no allergies) had mild respiratory illness (shortness of breath or rhinitis and coughing). Although the study was conducted in the summer, 8 (20%) of the remaining 40 *C. psittaci*–negative owners reported rhinitis or coughing during the past 2 weeks, some (7.5%) with a sore throat. Thus, statistical conclusions on the relationship of viable chlamydophila in humans and respiratory disease were not obtained.

The 23-year-old veterinary student was negative for *C. psittaci* before the study. His first pharyngeal specimen after he visited breeding facilities was positive by both PCR and culture. He remained infected until 1 month after ending his study but showed only mild respiratory illness.

C. psittaci genotype A was found in 5 pet bird owners and the veterinary student; these persons had rhinitis and a cough. The strain isolated from the pet bird owner who reported continued shortness of breath was genotype E/B. Thus, in contrast to the recently published reports on human psittacosis and pet birds (*9–11*), severe clinical signs with pneumonia did not occur in these patients and none were treated. However, 10 (25.6%) of 39 bird owners mentioned in the questionnaire that they had pneumonia after keeping Psittaciformes as pets, which was higher than the yearly rate of 8/1,000 pneumonia cases in Belgium. It is likely that pet bird owners and veterinarians are regularly infected and protected against severe disease. However, whether they become carriers and the possible consequences of infection are unknown. Protective clothing, including air filter face masks, is recommended for preventing occupational disease.

Conclusions

In our study, 18 (46.2%) of 39 breeding facilities had treated their birds with tetracycline, doxycycline, or enrofloxacin in the past year. Four (10.2%) of 39 also used tetracyclines prophylactically. Of these 18 facilities, 8 (44%) were positive for *C. psittaci* by nested PCR/EIA and 3 (16.6%) were positive by culture. Because of the risk of developing drug-resistant strains, as described for *Chlamydia suis* (*12,13*), regular use of antimicrobial drugs must be avoided. Since there is no vaccine against psittacosis, pet bird owners frequently use tetracyclines for treatment or prevention of respiratory disease. These drugs are sold on the Internet without a prescription because a prescription is not needed in every country. Before the advent of antimicrobial drug treatment, human mortality rates were 15%–20%. Thus, a vaccine and information on sensible use of antimicrobial drugs in Psittaciformes are needed to prevent psittacosis in humans and development of drug-resistant bacterial strains.

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Dr Vanrompay is a professor in the Faculty of Bioscience Engineering, Department of Molecular Biotechnology, Ghent University. Her primary research interests include *Chlamydiaceae* host-cell interactions, diagnosis, epidemiology, vaccine development, and animal models for studying protective immune responses after *C. psittaci* or *C. trachomatis* infections.

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Address for correspondence: Daisy Vanrompay, Faculty of Bioscience Engineering, Department of Molecular Biotechnology, Ghent University, Coupure Links 653, 9000 Ghent, Belgium; email: daisy.vanrompay@ugent.be

Rickettsia parkeri in Brazil

Iara Silveira,* Richard C. Pacheco,* Matias P. J. Szabó,† Hernani G. C. Ramos,* and Marcelo B. Labruna*

We report finding *Rickettsia parkeri* in Brazil in 9.7% of *Amblyomma triste* ticks examined. An *R. parkeri* isolate was successfully established in Vero cell culture. Molecular characterization of the agent was performed by DNA sequencing of portions of the rickettsial genes *gltA, htrA, ompA,* and *ompB*.

The first reported infection with *Rickettsia parkeri* was in *Amblyomma maculatum* ticks in Texas >65 years ago (*1*). Although its pathogenicity for humans was suspected or speculated during the following decades (*2*), *R. parkeri* was only recently recognized as a human tickborne pathogen (*3*). Extensive cross-reactivity exists among spotted fever group rickettsiae—especially *R. rickettsii* (the etiologic agent of Rocky Mountain spotted fever [RMSF] and Brazilian spotted fever [BSF])—and *R. parkeri.* Most of the time, *R. rickettsii* antigen is the only antigen used in serologic analysis for routine diagnosis of RMSF and BSF. Thus, many human cases of *R. parkeri* infection may be routinely misidentified as RMSF (*2*).

During the 1990s in Uruguay, several human cases of a tickborne rickettsiosis were diagnosed on the basis of serologic analyses; the spotted fever group organism *R. conorii* was used as antigen (*4*). Because *R. conorii* has never been found in the Western Hemisphere, another spotted fever group rickettsia may have been responsible for the reported cases (*5*). Because of recent reports of *R. parkeri* infection among *A. triste* ticks in Uruguay (where *A. triste* is the most common human-biting tick), this rickettsia has been suggested as the most probable agent of the Uruguayan spotted fever rickettsiosis (*5,6*). These data are corroborated by similar clinical findings found for both the American spotted fever caused by *R. parkeri* and Uruguayan spotted fever (*2,4*). *R. parkeri* has been reported only in the United States and Uruguay. We report *R. parkeri* infection of *A. triste* ticks in Brazil.

The Study

A. triste ticks were collected in a marsh area (21°07′06.7″S, 51°46′06.5″W) in Paulicéia County, state of São Paulo, Brazil. This area harbors a natural population of *A. triste,* mostly in the natural marsh environment along the Paraná River (*7*). Marsh deer (*Blastocerus dichotomus*) have been implicated as primary hosts for the adult stage of *A. triste* in the area, but the hosts for the immature stages of the tick remain unknown (*7*).

In January 2005, free-living adult *A. triste* ticks were collected by use of dry ice traps. Collected ticks were taken alive to the laboratory, where they were screened for rickettsial infection by using the hemolymph test with Gimenez staining (*8*). Immediately after hemolymph was collected, the ticks were stored at –80°C until used for further testing.

Ticks with hemolymph test results positive for infection with a *Rickettsia*-like organism were processed for isolation of *Rickettsia* in cell culture by using the shell vial technique (*9*). In brief, Vero cells were inoculated with tick body homogenate and incubated at 28°C. The level of cell infection was monitored by Gimenez staining of scraped cells from the inoculated monolayer; a rickettsial isolate was considered established after 3 passages, each reaching >90% of infected cells (*9*).

For cell isolation, a sample of 100%-infected cells from the fourth Vero cell passage was subjected to DNA extraction and thereafter tested by a battery of PCRs by using previously described primer pairs that targeted fragments of the rickettsial genes *gltA, htrA, ompA,* and *ompB* (*10*). Amplified products were purified and sequenced (*9*) and then compared with National Center for Biotechnology Information (NCBI) nucleotide BLAST searches (www.ncbi.nlm.nih.gov/blast).

Tick specimens with hemolymph test results negative for *Rickettsia*-like were thawed and individually processed for DNA extraction by the guanidine isothiocyanate–phenol technique (*11*). PCR amplification of a rickettsial gene fragment (398 nt) of the citrate synthase gene (*gltA*) was attempted on DNA from each tick by using the primers CS-78 and CS-323, which were designed to amplify DNA from all known *Rickettsia* spp. (*9*). Tick samples shown by PCR to be positive were tested further by a second PCR, which used the primers Rr190.70p and Rr190.602n, which amplify a 530-nt fragment of most of the spotted fever group *Rickettsia* (*12*). PCR products of the expected sizes were purified and sequenced (*9*) and then compared with NCBI nucleotide BLAST searches.

A total of 31 adult specimens of *A. triste* ticks were collected in January 2005. Specimens from 3 of the 31 ticks contained *Rickettsia*-like organisms, as determined by the hemolymph test. PCR amplification of the remaining 28 tick specimens was negative for *Rickettsia* spp. A *Rickettsia* organism was successfully isolated from only 1 of the 3 ticks with positive hemolymph test results. The isolate, designated as At24, was successfully established in Vero cell culture. PCR performed on DNA extracted from infected cells yielded the expected PCR products for all reactions. After DNA sequencing, the generated sequences of 1093, 489, 479, and 775 nt for the *gltA, htrA, ompA,*

*University of São Paulo, São Paulo, Brazil; and †Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil

and *ompB* genes, respectively, showed 100%, 99.8%, 100%, and 100% identity to corresponding sequences of *R. parkeri* Maculatum strain from the United States (GenBank accession nos. U59732, U17008, U43802, AF123717, respectively). Isolation attempts for the other 2 ticks with positive hemolymph test results were lost because of bacterial or fungal contamination. Nevertheless, remnants of ticks used to inoculate Vero cells were subjected to DNA extraction and tested by PCR for the *gltA* and *ompA* genes, as described above for ticks. Expected products were obtained from these PCR studies, and the generated sequences were 100% identical to the corresponding sequences of *R. parkeri* Maculatum strain (GenBank accession nos. U59732 and U43802, respectively). The frequency of *R. parkeri* infection among ticks examined in this study was 9.7% (3/31). Partial sequences (*gltA, htrA, ompA, ompB*) from *R. parkeri* strain At24 generated in this study were deposited into GenBank and assigned nucleotide accession nos. EF102236–EF102239, respectively.

Conclusions

Our report of *R. parkeri* infection of ≈10% of *A. triste* ticks from 1 area in the state of São Paulo highlights the possibility of *R. parkeri* causing human cases of spotted fever rickettsiosis in Brazil. However, in contrast to Uruguay, Brazil appears to have rare occurrences of *A. triste* and has never had a report of an *A. triste* bite in humans. In addition, no human case of spotted fever has been reported from sites within the known distribution area of *A. triste* in Brazil. On the other hand, an *R. parkeri*–like agent (strain Cooperi) was recently reported to have infected *A. dubitatum* ticks from a BSF-endemic area in São Paulo (*9*). Since *A. dubitatum* is a human-biting tick that is highly prevalent in many BSF-endemic areas (*13*), it is a potential candidate for transmission of *R. parkeri* to humans.

Spotted fevers caused by *R. parkeri* and by *R. rickettsii* differ in 2 ways: an eschar frequently occurs at the tick bite site in spotted fever cases caused by *R. parkeri*, and lymphadenopathy occurs in cases caused by *R. parkeri.* Because clinical descriptions of BSF (diagnosed solely by serologic testing that uses *R. rickettsii* antigen) with these specific clinical signs have been described recently in Brazil (*14,15*), human infections with *R. parkeri* may be occurring in this country. These clinical descriptions were from areas with large populations of *A. dubitatum* but no known occurrence of *A. triste.* Moreover, because *R. rickettsii* antigen has been the only antigen regularly used for diagnosis of BSF, human spotted fever cases due to *R. parkeri* or other spotted fever group rickettsiae may be misidentified as BSF in Brazil.

Our study demonstrated an exact concordance between ticks that were positive for *Rickettsia*-like organisms by the hemolymph test and those that were positive for rickettsial DNA by PCR. Previous studies in our laboratory (*9–11*) have demonstrated the same results or a slightly higher sensitivity of PCR for detection of rickettsiae in ticks.

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Dr Silveira is a PhD student at the University of São Paulo. Her research interests have focused on the ecology of tickborne diseases.

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Address for correspondence: Marcelo B. Labruna, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil 05508-270; email: labruna@usp.br

Tickborne Encephalitis, Southwestern France

Bruno Herpe,* Isabelle Schuffenecker,† Jérome Pillot,* Denis Malvy,* Benjamin Clouzeau,* Nam Bui,* Frederic Vargas,* Didier Gruson,* Hervé Zeller,† Marie E. Lafon,* Hervé Fleury,* and Gilles Hilbert*

We report an autochthonous human case of tickborne encephalitis (TBE) in the Bordeaux area, southwestern France. The patient was a farmer who had severe encephalomyelitis. ELISA and neutralization assay of serum and cerebrospinal fluid established the diagnosis. This potential new endemic focus for TBE virus should be further investigated.

Tickborne encephalitis (TBE) is the most important human arboviral infection of the central nervous system in Europe and Russia (*1*,*2*). The disease is endemic to areas where transmission vectors (*Ixodes ricinus*, *I. persulcatus*) are distributed. During the past 10 years, the incidence of the disease has increased, particularly in Lithuania, Germany, Switzerland, and Poland, and human cases have been reported from new areas (*3–5;* see also www.tbe-info.com/tbe.aspx). Tickborne encephalitis viruses (TBEV), such as dengue, yellow fever, West Nile, and Japanese encephalitis viruses, belong to the family *Flaviviridae*. Among the TBEV, 3 genotypes have been described (*6*): the European subtype transmitted by *I. ricinus* and the Siberian and Far Eastern subtypes transmitted by *I. persulcatus*. Large mammals, such as goats, sheep, and cattle, are important blood-feeding hosts for adult ixodid ticks. Because the virus is excreted in milk, small outbreaks may result from consumption of raw milk from sheep or goats. To our knowledge, only viruses of the western genotype have been isolated in Western Europe. In central and Western Europe, cases occur between April and November, and peak in June–July and September–October in relation to tick activity (*1*,*2*). The progression of incidence follows the development of tourism, trekking, and camping/hiking in virus-endemic countries (*3*). The European subtype virus usually produces a biphasic illness. The incubation period lasts an average of 7–14 days (range 2–28 days). Primary infection is generally associated with a flulike syndrome, but infection may be asymptomatic (40% of cases). In 5%–30% of clinical cases, a second neurologic phase may occur with aseptic meningitis (50% of the cases), meningoencephalitis (40%), or meningoencephalomyelitis (10%) (*1*,*2*,*7*,*8*). The Far Eastern subtype TBEV infections are considered to be associated with more severe cases (mortality rate 10%–20%) and more frequent neurologic sequelae (5%–30%) (*1*). Specific diagnosis depends essentially on the detection of immunoglobulin M (IgM) antibodies in serum or cerebrospinal fluid (CSF) by ELISA (*9*).

In France, 5–10 cases of TBE are reported each year. Cases have been mainly reported since 1968 from Alsace-Lorraine in northeastern France (*2*). We report here the first, to our knowledge, autochthonous case from the Aquitaine region in southwestern France.

The Case

On May 28, 2006, a 70-year-old farmer, living in the area of Bordeaux, southwestern France, was admitted, febrile and comatose, to the intensive care unit of the "Pellegrin hospital" in Bordeaux. He had no relevant medical history and had not traveled abroad during the previous year. The fever had begun 36 hours earlier, accompanied by headache, nausea, and vomiting. An attached tick had been removed from patient's thigh during the previous week. He had no history of unpasteurized milk consumption. Physical examination found a temperature of 38.5°C and tachycardia. The patient had an altered consciousness with a Glasgow Coma Score of 7. He exhibited normal osteotendinous and cranial nerve reflexes, had no Babinski sign but had notable nuchal rigidity. Assisted ventilation was needed. A blood sample was collected at admission and showed the following: leukocyte count 10,300/mm^3 (neutrophils 7,180/mm^3, lymphocytes 1,140/mm^3); hemoglobin 14.2 g/L, platelet count 209,000/mm^3, creatinine 158 μmol/L, prothrombin rate 94%. The cerebrospinal fluid (CSF) findings were consistent with viral meningoencephalitis: leukocyte count 620 cells/mm^3, 45% lymphocytes, protein 1.25 g/L, glucose 0.6 g/L. The patient was treated with 12 g/day intravenous amoxicillin and acyclovir. On day 4, the Glasgow Coma Score was 15, but the patient exhibited hypotonic tetraplegia with no osteotendinous reflexes. Magnetic resonance imaging showed cervicoarthrosis myelopathy but no sign of myelitis. An electromyograph confirmed the diagnosis of peripheral polyneuropathy. Ten days later, rapid motor improvement of lower extremities was observed, and the patient was transferred on day 15 to a rehabilitation center. An incapacitating brachial diplegia with amyotrophy persisted after 6 months.

For diagnostic purpose, paired serum samples were analyzed. No IgM or IgG antibodies were detected against *Borrelia* spp., *Leptospira* spp., *Mycoplasma* spp., *Rickettsia* spp., *Brucella* spp., *Treponema pallidum,* HIV, hepatitis B

*University Hospital of Bordeaux, Bordeaux, France; and †Institut Pasteur, Lyon, France

virus, and hepatitis C virus. Results of PCR assays of CSF were negative for *Borrelia* spp., Enterovirus, Adenovirus, varicella-zoster virus, herpes simplex virus, Epstein-Barr virus, and cytomegalovirus. IgM capture and IgG indirect in-house assays with native antigen (from Hypr 1953 TBEV strain, kindly provided by F. Heinz) were performed to detect IgM and IgG TBEV antibodies. The cut-offs were as follows: IgG cut-off 0.05; IgM cut-off 0.2. An 80% plaque-reduction neutralization test ($PRNT_{80}$) was performed by using the Hypr 1953 strain to determine the titer of TBEV neutralizing antibodies. Rising TBEV IgG optical density values were detected in a single assay between the earliest and the latest serum specimen (Table). High titers of TBEV neutralizing antibodies were detected on days 11 and 24 after clinical onset of disease (Table). A nested reverse transcription–PCR (*10*) for TBEV in early serum and CSF (days 3 and 4) showed negative results. No TBEV could be isolated by culture on Vero E6 cells.

Conclusions

We describe, to our knowledge, the first case of tickborne encephalitis in southwestern France. High levels of TBEV IgM were observed in serum and CSF samples, as is usually observed in TBE neurologic cases (*9*). In addition, the high titers of TBEV-specific antibodies, determined by PRNT, reinforces the conclusion that TBEV is the probable cause of the encephalitis-like condition of the patient. However, no virus was isolated and no genome detected. Early CSF and serum specimens had not been immediately tested for TBEV, and the successive freezing and thawing may explain the negative results. Another explanation could be that the first phase of the disease was not recognized by the patient and that the viremic phase was already finished when the patient was hospitalized, as suggested by the presence of high titers of TBEV-neutralizing antibodies on day 8 after admission. This explanation is compatible with the chronic exposure of the farmer to tick bites. As a differential diagnosis, infection by *Borrelia garinii*, which is also transmitted by *I. ricinus*, could be excluded because no specific IgM and IgG were detectable by serologic testing and no dermatologic signs characteristic of Lyme disease were visible. However, 2 other tickborne viruses, genetically related to TBEV, could not be formally excluded: Louping ill virus and Spanish sheep encephalitis virus.

To date, in France most TBE cases have been reported from Alsace-Lorraine. However, in 2003, 3 cases were reported from the French Alpen region (*5*), likely linked to goat cheese consumption. These new cases raised the question of the extension of TBEV that is endemic in France, as has been observed in Germany and Switzerland. Since 2003, however, patients from this region with meningitis and encephalitis have been more systematically screened for TBEV, and no other case has been identified (I. Schuffenecker, unpub. data).

The discovery of the first TBE case in southwestern France raises the question of the mode of emergence of the virus in this region. The viral cycle involves mainly rodents or deer and ticks with humans as accidental hosts. Domestic ruminants act more as tick transporters than as a reservoir. One hypothesis to the emergence could be the introduction of infected ticks through animal transportation or bird migration. A field survey in the close vicinity of the farmer's house and fields could yield valuable results. Collecting ticks and rodents could provide the opportunity to identify the circulating strains (as recently was done in Finland and Estonia) (*11*). Also, a seroprevalence study on domestic animals could provide information on the level of circulation of flaviviruses.

Finally, TBEV should be more systematically screened for in patients with encephalitis and meningitis in the absence of any other etiologic diagnosis. Because this finding has implications for expanding vaccine coverage to forestry and agriculture workers, additional epidemiologic data about TBEV circulation in southwestern France should be obtained.

Dr Herpe is a clinical fellow in the Department of Medical Intensive Care at the University Hospital of Bordeaux, France. His research interests include infectious and tropical diseases.

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Table. TBEV diagnosis results*†

Samples	Days after clinical onset	ELISA TBEV IgM (OD value)	ELISA TBEV IgG (OD value)	$PRNT_{80}$ TBEV antibody titers	TBEV RT-PCR (*10*)	Virus culture
CSF	3	**1.15**	ND	ND	Negative	ND
CSF	4	ND	ND	ND	Negative	Negative
Serum	4	**1.07**	**0.07**	ND	Negative	Negative
Serum	11	**1.58**	**0.4**	**640**	ND	ND
Serum	24	**1.65**	**1.82**	**640**	ND	ND

*TBEV, tickborne encephalitis virus; IgM, immunoglobulin M; OD, optical density; PRNT80: plaque-reduction neutralization test (80%); RT-PCR, reverse transcription–PCR; CSF, cerebrospinal fluid; ND, not done. **Boldface** type indicates positive values.
†IgM capture and IgG indirect in-house assay were performed to detect IgM and IgG TBEV antibodies. The cut-offs were as follows: IgG cut-off 0.05; IgM cut-off 0.2. A PRNT was performed using the Hypr TBEV strain to determine the titer of TBEV-neutralizing antibodies.

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Address for correspondence: Bruno Herpe, Division of Medical Intensive Care, University Hospital of Bordeaux, 9 Place Amélie Raba Léon, 33076 Bordeaux, France; email: bruno.herpe@chu-bordeaux.fr

Cell Culture Assay for Human Noroviruses

To the Editor: We read with great interest the article on human norovirus (hNoV) by Straub et al. (*1*). By using 3-dimensional aggregates of a highly differentiated intestinal epithelial cell line, the investigators claimed to have established an in vitro cell culture model that "support[s] the natural growth of human noroviruses." While the authors provide compelling evidence of successful virus infection through microscopy, hybridization of viral RNA after 5 passages in cell culture, and preliminary evidence of viral RNA replication through limiting dilution PCR, we question the level of virus replication that is actually achieved in this system.

Straub et al. demonstrate through fluorescent in situ hybridization the presence of viral RNA through 5 passages in his system. This phenomenon could be similar to the findings of Duizer et al. (*2*), if the level of replication simply maintained the viral titer. Therefore, we argue that virus replication curve, estimated by using quantitative real-time PCR or semiquantitative endpoint dilution PCR with the end-dilution of each sample from different time points in this system, will conclusively determine the suitability of this model as a productive virus replication system. To support our hypothesis, we point to the pig model for hNoV infectivity (*3*). In that study investigators failed to observe an increase in viral shedding from symptomatic piglets upon serial passage, despite successful intracellular detection of viral RNA and newly synthesized virus-encoded protein in host cells dying of apoptosis. This suggests that the demonstration of cytopathic effect and virus internalization in cells alone may not provide direct evidence of productive virus replication. In conclusion, although we acknowledge that Straub et al. have provided evidence of successful hNoV infection in vitro, we suggest subsequent studies to characterize the level of virus replication in this system.

Martin C.W. Chan,* Y.P. Wong,* and Wai K. Leung*

*The Chinese University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China

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Address for correspondence: Wai K. Leung, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China; email: wkleung@cuhk.edu.hk

In Response: We appreciate the comments provided by Chan et al., in response to our recently published article (*1*). The specific aim of our project was to develop an in vitro cell culture infectivity assay for human norovirus (hNoV) to enhance risk assessments when these viruses are detected in water supplies. Reverse transcription (RT) qualitative or quantitative PCR are the primary assays for waterborne hNoV monitoring. However, these assays cannot distinguish infectious from noninfectious virions. When hNoV is detected in water supplies, information provided by our infectivity assay will improve risk assessment models and protect human health, regardless of whether we are propagating hNoV. Indeed, in vitro cell culture infectivity assays for the waterborne pathogen *Cryptosporidium parvum* that supplement approved fluorescent microscopy assays do not result in amplification of the environmentally resistant hard-walled oocysts (*2*). However, identification of life cycle stages in cell culture provides evidence of infectious oocysts in a water supply.

Nonetheless, Leung et al.'s assertion regarding the suitability of our method for the in vitro propagation of high titers of hNoV is valid for the medical research community. In this case, well-characterized challenge pools of virus would be useful for developing and testing diagnostics, therapeutics, and vaccines. As further validation of our published findings, we have now optimized RT quantitative PCR to assess the level of viral production in cell culture, where we are finding increases in viral titer. The magnitude and time course of these increases is dependent on both virus strain and multiplicity of infection. We are currently preparing a manuscript that will discuss these findings in greater detail, and the implications this may have for creating viral challenge pools.

Timothy M. Straub,*
Kerstin Höner zu Bentrup,†
Patricia Orosz-Coghlan,‡
Alice Dohnalkova,*
Brooke K. Mayer,*[1]
Rachel A. Bartholomew,*
Catherine O. Valdez,*
Cynthia J. Bruckner-Lea,*
Charles P. Gerba,‡
Morteza A. Abbaszadegan,§
and Cheryl A. Nickerson†[1]

*Pacific Northwest National Laboratory, Richland, Washington, USA; †Tulane University School of Medicine, New Orleans, Louisiana, USA; and ‡University of Arizona, Tucson, Arizona, USA

[1]Current affiliation: Arizona State University, Tempe, Arizona, USA

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Address for correspondence: Timothy M. Straub, Pacific Northwest National Laboratory – Chemical and Biologic Sciences, PO Box 999, MS P7–50, Richland, WA 99352, USA; email: timothy.straub@pnl.gov

Anthrax in Red Deer (*Cervus elaphus*), Italy

To the Editor: Anthrax is hypoendemic in Italy; a few outbreaks occurred yearly involving unvaccinated herbivores on pastures in central and southern regions and the major islands. Multiple-locus variable-number tandem-repeat analysis (MLVA) with 8 variable-number tandem repeats (VNTRs) of Italian isolates of *Bacillus anthracis* has identified 9 genotypes belonging to cluster A1a (*1*). An isolate of cluster A3 has been identified recently in Sardinia, which suggests that such a strain could have been introduced into Italy from another country (*1*).

A total of 37 anthrax outbreaks occurred in a 41-day period from August 28 to October 3, 2004, in a restricted area of Pollino National Park (Basilicata region in southern Italy) and resulted in the deaths of 124 domestic or wild animals. Two suspected cases of cutaneous anthrax in humans were recorded. Pollino National Park contains several species of feral animals. Since 1990, there has been a program for reintroduction of red deer (*Cervus elaphus*) into this park from Tuscany, Italy, and Carinthia, Austria. The animals are kept in quarantine in a corral by the veterinary services of the park and given an electronic tag before their release. At the time of the anthrax outbreaks, the red deer population of the park was 45, of which 10 were living in the corral. These outbreaks killed 8 deer (4 free-ranging and 4 confined animals).

Each carcass was examined by the veterinary officer, who collected clinical samples that were examined for *B. anthracis* by using standard procedures of the Istituto Zooprofilattico Sperimentale of Puglia and Basilicata. DNA from the suspected colonies was analyzed by PCR with primers specific for *B. anthracis* (*2*) and subsequent genotyping by using MLVA with 8 VNTRs (*3*). All *B. anthracis* isolates belonged to cluster A1a, genotype 1 (A. Fasanella, unpub. data). This genotype was also identified in subsequent outbreaks that involved farm animals in the same area and resulted in the deaths of 116 domestic animals, including 81 cattle, 15 sheep, 9 goats, and 11 horses. Red deer showed the highest mortality rate during these outbreaks (Table). An ELISA (*4*) performed with 27 serum samples obtained from deer in the park detected low levels of antibodies to *B. anthracis* in 22% of the examined animals. This seroprevalence is consistent with levels found in unvaccinated livestock reared in areas endemic for anthrax (A. Fasanella, unpub. data).

A vaccination program was then instituted for farm animals, but the deer population in the park was excluded because no experimental data were available on the safety and efficacy of Carbosap vaccine (Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Foggia, Italy) in wild ruminants. Extensive vaccination limited the outbreaks in livestock and red deer, which probably prevented further spread of infection from farm animals to free-ranging deer.

These anthrax outbreaks in southern Italy suggested that red deer are highly susceptible to infection with *B. anthracis* and that the mortality rate in these deer could be even higher than that observed in domestic animals. Although epidemiologic data are limited and need to be supported experimentally by assessment of the 50% lethal dose of *B. anthracis* in red deer, the ecologic effect on deer populations in parks should not be underestimated. Moreover, concerns for public health may arise in parks in disease-endemic areas, where susceptible wild animals could represent an amplification factor for *B. anthracis* spores, which increases the probability of outbreaks in domestic animals and in humans living near, working in, or visiting the parks. This article stresses the need for evaluating the safety and efficacy of *B. anthracis* vaccines in deer and for including wild ruminants in the anthrax prophylaxis programs.

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Antonio Fasanella,*
Lucia Palazzo,*
Antonio Petrella,*
Vincenzo Quaranta,*
Bruno Romanelli,*
and Giuliano Garofolo*

Table. Mortality rates during anthrax outbreaks, Italy, 2004

Animal	Population of area	No. (%) dead animals
Cattle	≈7,000	81 (≈1.15)
Sheep	≈20,000	15 (≈0.075)
Goats	≈13,000	9 (≈0.069)
Horses	≈600	11 (≈1.83)
Red deer	45	8 (≈17.77)

*Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Foggia, Italy

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Address for correspondence: Antonio Fasanella, Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Anthrax Reference Institute of Italy, Via Manfredonia, 20, 71100 Foggia, Italy; email: a.fasanella@izsfg.it

Invasive Freshwater Snail, China

To the Editor: *Pomacea canaliculata*, an invasive freshwater snail native to South America, was first introduced as a food to Taiwan in1979 and then to Mainland China in 1981 (*1*). It adapted well to the environment, particularly to the southern parts of the Mainland, spreading rapidly to more than 10 provinces (Figure) and causing tremendous damage to agriculture and the ecosystem (*1*,*2*). Thousands of hectares of rice, vegetables, and other crops in these provinces were destroyed (*2*).

Even more alarming were the multiple outbreaks of a severe brain disease (angiostrongyliasis) in Taiwan that were linked to *P. canaliculata (3,4)*. Angiostrongyliasis is caused by *Angiostrongylus cantonensis*, a lung nematode of wild rodents, commonly known as the rat lungworm. In Mainland China, epidemiologic evidence also indicates that *P. canaliculata*, because of its high susceptibility to *A. cantonensis*, is becoming the most important natural intermediate host for this parasite *(5)*. Previously, other terrestrial snails like *Achatina fulicia*, and some species of slugs such as *Philomycus bilineatus* were regarded as the major intermediate hosts for *A. cantonensis* (*6*). Epidemiologic survey results from 1997 to 1999 demonstrated that 20.8%–69.4% of *P. canaliculata* were infected with *A. cantonensis* in some regions of Guangdong, Zhejiang, and Fujian Provinces (*5*). Even in provinces where the snail is not found, a high incidence and prevalence of infection occur because of its widespread distribution, high susceptibility to *A. cantonensis*, and growing popularity as a food. In 1997, 2002, and 2002, ingestion of raw or undercooked *P. canaliculata* meat led to 3 outbreaks of angiostrongyliasis infecting >100 patients (*6,7*). A 2006 outbreak in Beijing infected 131 persons *(8)*. Based on the biologic characteristics of *P. canaliculata*, blocking its life cycle is one of the most effective methods to limit the outbreak of angiostrongyliasis. However, the current widespread distribution of *P. canaliculata* in China and the lack of a highly effective control method make the disease extremely difficult to eliminate (*9*). More outbreaks associated with ingestion of this snail will likely occur if food safety rules are not strictly enforced. Citizens must also be educated to avoid eating raw, undercooked snail meat or raw vegetables from regions that may be contaminated with infective mucous trails deposited by these snails (*10*).

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Figure. Distribution of *Pomacea canaliculata* in China. The dark triangles indicate the regions where angiostrongyliasis outbreaks were reported due to ingestion of raw or undercooked *P. canaliculata* snails.

Qiao-Ping Wang,*
Xiao-Guang Chen†
and Zhao-Rong Lun*

*Sun Yat-Sen (Zhongshan) University, Guangzhou, People's Republic of China; and †Southern Medical University, Guangzhou, People's Republic of China

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Address for correspondence: Zhao-Rong Lun, Center for Parasitic Organisms, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen (Zhongshan) University, Guangzhou 510275, People's Republic of China; email: lsslzr@mail.sysu.edu.cn

Possible Avian Influenza (H5N1) from Migratory Bird, Egypt

To the Editor: Wild migratory birds are reservoirs for low pathogenic avian influenza (LPAI) viruses (*1*), but their role in transmitting highly pathogenic avian influenza (HPAI) viruses is hotly debated and unclear (*2*–*4*). Beginning in July 2005, a clade of HPAI (H5N1) viruses rapidly expanded from an apparent focus in western People's Republic of China and spread to the Middle East, Africa, and Europe (*5*). Genetic analysis of HPAI virus isolates from dead wild birds along major flyways indicated that the strains were closely related to the Qinghai H5N1 A/bar-headed goose/Qinghai/65 /2005 virus (clade II) (GenBank accession no. DQ095622). In addition to transmission to domestic poultry, HPAI (H5N1)–infected mute swans have been implicated in direct transmission to humans in Azerbaijan (*6*).

The US Naval Medical Research Unit No. 3 and the Ministry of Environment of Egypt have collaborated since 2003 in obtaining samples from migratory birds to detect circulating influenza viruses. During the 2005–06 migratory birds season, 1,304 migratory birds were sampled from either live bird markets or cage birds trapped by fishermen in Port Said, Damietta, Fayoum, Arish, and Sharm el Sheikh (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/13/7/1120-appG.htm).

A total of 203 cloacal swab samples were positive for influenza A virus matrix gene when tested by real-time PCR, and 2 were also positive for the hemagglutinin 5 (H5) gene by using specific primers (*7*). Of the 2 migratory birds positive for the H5 gene, the first was a common teal (*Anas crecca*) captured in the Nile Delta region of Damietta in October 2005 (online Appendix Figure, panel A). Sequencing of the H5 gene showed that this virus was an LPAI most closely related to strain A/mallard/Bavaria/1/2005(H5N2) (GenBank accession no. DQ387854 (*2*).

In January 2006, an influenza A H5 virus (weak positive result) was detected in another common teal (trapped in a cage by a fisherman) sampled from the Damietta region in December 2005 (online Appendix Figure, panel A). The low viral load, coupled with the failure to isolate the virus, precluded the laboratory from conducting sequence analysis at the time on the basis of insufficient template material. After the outbreak of influenza A (H5N1) in poultry and humans in Egypt in February 2006, additional retrospective attempts to concentrate RNA were used to assess potential introduction scenarios. After multiple RNA extractions were conducted and the RNA was concentrated, this specimen was found to be positive for the neuraminidase 1 (N1) gene by real-time PCR.

The hemagglutinin gene from both teal strains was sequenced (≈1,596 bp). Sequences were aligned with other influenza A (H5N1) strains from Egypt (9 from humans, 5 from chickens). Twenty other strains with high similarity and from different locations were selected by using a GenBank search algorithm and included in the alignment. A phylogenetic analysis was conducted by using the Kimura 2-parameter model. The LPAI H5 virus strain was used as an outgroup in a neighbor-joining phylogenetic tree. Bootstrap analysis with 500 replicates of sequence data was also conducted by using MEGA 3.1 software (*8*).

Phylogenetic analysis showed clustering of the HPAI (H5N1) strains collected from 1 geographic region (country) (online Appendix Figure, panel B). All HPAI (H5N1) strains from Egypt from humans or chickens analyzed clustered with a bootstrap support value of 98%. Furthermore, the A/Teal/Egypt/14051-NAMRU3/2006

(H5N1) strain (collected in December 2005; online Appendix Figure, panel A) is an HPAI and is closely related to the parent of the group of viruses isolated in the early 2006 Egypt outbreak, with an average identity of 99.4% with all other strains from Egypt and a bootstrap support value of 96% (online Appendix Figure, panel B). Despite the rapid spread of this clade (Qinghai-like strain) to many countries, since late 2005, strains analyzed in this study showed low-level genetic variation (<2%).

Brown et al. reported that species can vary greatly in their response to HPAI (*9*). At least in ducks, it appears that viral shedding is highest in birds with clinical signs of infection, and lowest, as seen in the common teal infected with the HPAI strain in this study, in birds with subclinical infections. These subclinical infections may be due to flock immunity from previous exposure to LPAI H5 virus or genetic factors. This suggestion is conceivable in light of the LPAI H5 virus detected in the other teal a few months earlier.

Such naturally resistant wild birds might serve as vectors for introduction of HPAI viruses into new locations. Data presented herein suggest that an HPAI virus may have been introduced into Egypt through a migratory bird. Whether poultry were infected before mid-February or the teal was infected with influenza A (H5N1) virus by a domesticated species is not unknown. The low degree of viral shedding indicates that detection of any influenza A (H5N1) virus in wild birds in a new region should be immediately followed up with efforts to characterize the virus to control the spread of new subtypes/strains of HPAI into new locations.

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Magdi D. Saad,*
Lu'ay S. Ahmed,†
Mohamed A. Gamal-Eldein,†
Mohamed K. Fouda,
Fouda M. Khalil,†
Samuel L. Yingst,*
Michael A. Parker,*
and Marshall R. Montevillel*

*US Naval Medical Research Unit No. 3, Cairo, Egypt; and †Ministry of Environment, Cairo, Egypt

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Address for correspondence: Magdi D. Saad, Commanding Officer, US Naval Medical Research Unit No.3, PSC 452, Box 5000, MDS Code 303, FPO AE 09835-0007, Cairo, Egypt; email: darwishm@namru3.med.navy.mil

Community-acquired Extended-Spectrum β-Lactamase Producers, United States

To the Editor: Extended-spectrum β-lactamase (ESBL)–producing organisms have become a common problem for patients in hospitals and other healthcare facilities (*1*). Community-onset ESBL infections have recently been described in Spain, the United Kingdom, Israel, and Canada (*2*,*3*). Typically, the infections are urinary tract infections (UTIs) with CTX-M–producing *Escherichia coli*. These organisms may be resistant to most or all antimicrobial agents commonly used to treat UTIs, such as ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, and ceftriaxone.

Although CTX-M–producing *E. coli* have previously been found in the United States (*4*), clinical descriptions of community-acquired ESBL-producing *E. coli* infections have not been reported in this country. We describe 2 healthy young women in Pennsylvania in whom UTI with CTX-M-15–producing *E. coli* developed.

A 25-year-old woman was seen in October 2006 at the emergency department of a hospital in Pittsburgh reporting frequent urination, chills, and bilateral back pain. She had no relevant past medical history except for previous UTIs. Results of a physical examination were unremarkable.

Urinalysis showed >20 leukocytes per high-powered microscopic field. Urine culture grew >100,000 colonies/mL *E. coli*, resistant to trimethoprim/sulfamethoxazole and ciprofloxacin. The organism was positive by phenotypic confirmatory tests for ESBL production. Molecular characterization showed a gene encoding the CTX-M-15 β-lactamase. The patient was treated empirically with oral ciprofloxacin, 500 mg every 12 hours for 10 days and was lost to further follow-up. She has not had urine or blood cultures collected through our healthcare system in the 3 months since the time of her UTI.

A 24-year-old woman visited a student health service in Pittsburgh in September 2006 with urinary frequency, nausea, and back pain. There was mild costovertebral angle tenderness, and a clinical diagnosis of early pyelonephritis was made. The patient had no relevant past medical history except for previous UTIs (3 in the last 12 months). She was treated empirically with oral ciprofloxacin, 500 mg every 12 hours for 7 days. Urine culture grew >100,000 colonies/mL *Streptococcus agalactiae* and 25,000 colonies/mL *Klebsiella pneumoniae*. The *K. pneumoniae* was not an ESBL producer and was resistant to ampicillin and susceptible to ciprofloxacin, trimethoprim/sulfamethoxazole, ceftriaxone, and tobramycin.

Ten days after the initial visit, the patient returned for further assessment, and a follow-up urine sample was collected. There was no pyuria, but urine culture grew 15,000 colonies/mL *E. coli*. The organism was an ESBL producer that was resistant to ciprofloxacin and tobramycin and susceptible to trimethoprim/sulfamethoxazole. Given the lack of pyuria and the low intensity of symptoms, the patient was not treated with antimicrobial agents. She has not returned for follow-up, and no urine or blood cultures have been collected through our healthcare system in the 3 months since the time of her *E. coli* UTI. Molecular characterization of the gene encoding the ESBL indicated that it encoded CTX-M-15.

To our knowledge, these 2 cases represent the first cases of community-acquired ESBL-producing *E. coli* known to have occurred in the United States. In 2003, Moland et al. detected CTX-M–like ESBLs in the United States (*4*). The 9 *E. coli* isolates they described were from patients in 5 states—Virginia, Idaho, Ohio, Washington, and Texas—which suggests that CTX-M producers are geographically widespread in this country. Although some isolates were from a urinary source, these isolates were from a hospital surveillance study and the authors were unable to determine if these organisms were from community-acquired infections.

These 2 patients did not appear to have substantial clinical effects from their infections. However, the potential importance of community-acquired ESBL-producing *E. coli* is that UTI may be associated with bloodstream infection. Empiric antimicrobial therapy of bloodstream infection presumed to be of urinary tract origin typically comprises use of fluoroquinolones, aminoglycosides, or ceftriaxone. ESBL-producing *E. coli* may be resistant to all of these antimicrobial agents. In the United Kingdom, 25 of the first 108 patients with documented community-onset ESBL-producing *E. coli* infections died (*5*). Frequent occurrence of ESBL-producing *E. coli* in the United States would be an important public health problem and may necessitate changes in empiric antimicrobial therapy.

In Europe, many community-onset infections with ESBL producers actually appear to be healthcare associated, rather than truly community acquired. Patients with these infections may have been hospitalized in the recent past or have had relevant underlying diseases (*2*,*6*). It is also possible that the 2 infections we observed were not actually community-acquired and that these 2 women had unrecognized exposure to hospitals or healthcare facilities. However, the CTX-M producers we have previously isolated from hospitals in Pittsburgh produced CTX-M-9, not CTX-M-15 (data not shown). Our healthcare system provides coverage for most people in Pittsburgh and surrounding regions, and we found no records of previous hospitalization or chronic healthcare contact for either of the 2 persons. As far as we can ascertain, the 2 cases we describe appear to be truly community acquired, as determined by previous definitions differentiating healthcare-associated from community-acquired infections (*7*).

If these infections were truly community-acquired, how and why did they arise? The CTX-M-15 ESBL has been found in many countries. We do not know the travel histories of these 2 patients. Thus, the organisms may have been acquired overseas. We assessed the genetic relatedness of these 2 strains by pulsed-field gel electrophoresis but found no evidence of clonality (data not shown). Another possibility is that food was the source of infections. CTX-M-15–producing *E. coli* have been detected in food-producing animals (*8*), and we have recently found CTX-M-15–producing *E. coli* in chicken sold at a Pittsburgh area supermarket. We are currently conducting ongoing surveillance for community-acquired ESBL producers in our region in *E. coli* isolates from both humans and from foodstuffs to determine the prevalence of CTX-M producers in the United States.

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Yohei Doi,* Jennifer Adams,* Alexandra O'Keefe,* Zubair Quereshi,* Lindsay Ewan,* and David L. Paterson*[1]

*University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

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Address for correspondence: David L. Paterson, Infectious Diseases Unit, University of Queensland, Royal Brisbane and Women's Hospital, Butterfield St, Herston, Queensland, QLD 4029 Australia; email: david.antibiotics@gmail.com

[1]Current affiliation: University of Queensland, Royal Brisbane and Women's Hospital, Herston, Queensland, Australia

Japanese Encephalitis Outbreak, Yuncheng, China, 2006

To the Editor: Japanese encephalitis (JE) epidemics have occurred only in Asia. More than 50,000 cases of JE with ≈10,000 deaths have been reported since 1998 (*1*,*2*). The People's Republic of China reported 5,104 cases and 214 deaths in 2005. Most of these deaths occurred in infants (*3*,*4*).

During July and August 2006, an outbreak of viral encephalitis occurred in Yuncheng, Shanxi Province, People's Republic of China. A total of 66 cases (1.32/100,000 population) were reported, including 19 deaths (case-fatality rate 28.8%). The cases had a widespread distribution over 9 counties and involved 37 towns and 61 administrative villages. The ratio of male-to-female patients was 1:0.89. A distinct clinical feature of this outbreak was the age distribution. More than 86% of the patients were >30 years of age, with only 10% of patients <7 years of age; ≈95% of the deaths occurred in patients >50 years of age (*5*).

We report serologic and virologic findings for the 2006 outbreak of viral encephalitis. Forty-six clinical specimens collected from 34 patients who had a diagnosis of viral encephalitis, including 33 serum samples and 13 cerebrospinal fluid (CSF) samples, were studied. All serum samples were screened for immunoglobulin M (IgM) to West Nile virus (WNV) by using the WNV IgM-capture ELISA kit (PanBio, Brisbane, Queensland, Australia) and for IgM to dengue virus or Japanese encephalitis virus (JEV) by using the JE-Dengue IgM Combination ELISA kit (PanBio). Results for JEV were confirmed by using the JE Virus IgM-Capture ELISA kit (Shanghai B & C Enterprise Development Co. Ltd, Shanghai, People's Republic of China).

WNV-specific or dengue virus–specific IgM was not detected in any samples. JEV-specific IgM was detected in 27 (80%) patients, which indicated recent JEV infections. The other 7 patients were negative for JEV by ELISA and reverse transcription–PCR (RT-PCR). Increases ≥4-fold in neutralizing antibodies were detected in acute- and convalescent-phase serum samples from 9 patients (10 serum pairs were collected during the outbreak).

Attempts were made to detect virus in CSF of patients and in 2,400 mosquitoes. Mosquitoes (mainly *Culex* spp.) were collected in cow sheds and hog pens around houses and processed into pools of 100. Total RNA was extracted from CSF or mosquito homogenate by using the QIAamp viral RNA extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's specifications. RT was performed by using Ready-To-Go-You Prime First Strand Beads (Amersham Pharmacia Biotech, Piscatawy, NJ, USA) and a seminested PCR to amplify 492-bp gene fragments of the premembrane (PrM) sequence of JEV by using the Takara LA Taq PCR kit (Takara Bio Inc., Shiga, Japan). The primers were derived from Ishikawa strain genome sequences (GenBank accession no. AB051292). Primers PrMF: 5′-CGT TCT TCA AGT TTA CAG CAT TAG C-3′ (251–275), PrMR1: 5′-CGY TTG GAA TGY CTR GTC CG-3′ (724–743), and PrMR2: 5′-CCY RTG TTY CTG CCA AGC ATC CAM CC-3′ (901–925) were used.

JEV PrM gene was amplified from CSF of 6 (46%) of 13 patients and 10 of 24 pools of mosquitoes by using the same seminested RT-PCR. To identify JEV genotype(s) involved in this outbreak, PCR products were sequenced. Eleven sequences (GenBank accession nos. EF434264–EF434274) were obtained from 6 patients and 5 pools of mosquitoes. The 11 sequences were compared phylogenetically with17 known JEV strains of the 4 recognized

genotypes (classified on the basis of a 240-nt region of the prM gene). As shown in the Figure, the 11 sequences were those of JEV.

Further analysis showed that these 11 sequences can be grouped into genotypes I and III. Both genotypes were found in patient and mosquito samples, indicating that these genotypes co-circulated during this JE outbreak.

JE has been endemic in Yuncheng for many years (*6*). A vaccine against JE (SA14–14–2) has been used in this area in infants, but not in adults. This might be 1 reason why a higher adult incidence was found in this outbreak. JEV genotype III had been the predominant genotype in previous years, but genotype I has been recently detected at increased frequencies (*7–10*). Detection of 2 JEV genotypes in 1 epidemic has not been reported. Whether simultaneous circulation of >1 genotype during an outbreak indicates a new type of emergence of JEV or that this has occurred and not been detected is unknown.

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Li-Hua Wang,* Shi-Hong Fu,* Huan-Yu Wang,* Xiao-Feng Liang,† Jing-Xia Cheng,‡ Hong-Mei Jing,§ Gen-Lao Cai,¶ Xing-Wang Li,# Wen-Yuan Ze, Xin-Jun Lv,* Hua-Qing Wang,† Ding-Lin Zhang,¶ Yun Feng,* Zun-Dong Yin,† Xiao-Hong Sun,* Tie-Jun Shui,† Ming-Hua Li,* Yi-Xing Li,† and Guo-Dong Liang***

*Institute for Viral Disease Control and Prevention, Beijing, People's Republic of China; †Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China; ‡Shanxi Center for Disease Control and Prevention, Taiyuan, People's Republic of China; §Yuncheng Center for Disease Control and Prevention, Yuncheng, People's Republic of China; ¶Yuncheng Infectious Diseases Hospital, Yuncheng, People's Republic of China. #Beijing Ditan Hospital, Beijing, People's Republic of China; and **Beijing Institute of Biological Products, Beijing, People's Republic of China

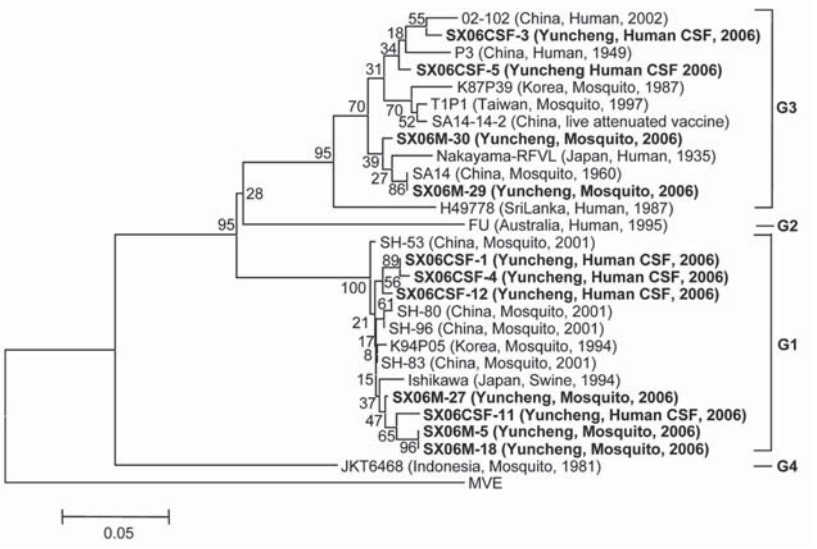

Figure. Phylogenetic analysis of Japanese encephalitis virus strains predicted from premembrane gene sequences. Neighbor-joining tree was generated by using MEGA 3.1 software (www.megasoftware.net) and rooted with Murray Valley encephalitis (MVE) virus sequence information. Bootstrap confidence limits for 1,000 replicates are indicated above each branch. Horizontal branch lengths are proportional to genetic distance; vertical branch lengths have no significance. Scale bar indicates no. nucleotide substitutions per site. All sequences from this study are in **boldface**. Genotypes are indicated on the right. Designations are listed first, followed by country, source, and year of isolation. CSF, cerebrospinal fluid.

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Address for correspondence: Guo-Dong Liang, State Key Laboratory for Infectious Disease Control and Prevention, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 100 Yingxin St, Xuanwu District, Beijing 100052, People's Republic of China, email: gdliang@hotmail.com

Chloroquine-Resistant *Plasmodium vivax*, Brazilian Amazon

To the Editor: *Plasmodium vivax* is the protozoan that causes the second most common form of malaria. Some resistant strains to chloroquine (CQ) occur in a few places in Asia and the Indo-Pacific Region (*1*–*4*). Although resistance of *P. vivax* to CQ has already been described in South America (*5*–*7*), there are limited data regarding this issue.

CQ plus primaquine is the standard treatment for vivax malaria worldwide. Presently, this drug regimen exhibits satisfactory efficacy in the Brazilian Amazon. However, in recent years several treatment failures presumably related to CQ resistance, have been reported in the city of Manaus (Amazonas) where vivax malaria predominates *(7)*. This observation warrants local attention despite these cases having no confirmation of CQ blood levels on the basis of the appearance of asexual parasites against CQ plus desethylchloroquine levels exceeding the minimally effective plasma concentration proposed for sensitive parasite strains (≥10 ng/mL) (*8*), according to Pan American Health Organization recommendations *(9)*.

From September 2004 to February 2005, a 28-day in vivo test was conducted at the Foundation for Tropical Medicine of Amazonas (FMTAM) in Manaus, Brazil, to assess the efficacy of standard supervised CQ therapy. The test involved 166 volunteers with uncomplicated vivax malaria. Each volunteer was administered uncoated, scored, 150-mg CQ tablets (10 + 7.5 + 7.5 mg/kg at 24-hour intervals) (*9*). Primaquine was withheld until day 28 (dose regimen of 30 mg/day for 7 days). Among the 109 volunteers who completed the in vivo test, 19 had positive blood smears within the 28-day follow-up (1 on day 14, 3 on day 21, and 15 on day 28). All were required to undergo alternative therapy (mefloquine). Adequate CQ absorption was confirmed in these cases on day 2 with a mean ± SD CQ plasma concentration of 785.4 ± 800.1 ng/mL) (*10*) Suspected therapeutic failure (*P. vivax* CQ resistance) was confirmed in 11 (10.1%) of 109 persons with a mean isolated choloroquine plasma concentration ≥10 ng/mL (356.6 ± 296.1 ng/mL) (*9*). Desethylchloroquine levels in plasma were not measured.

Previously, a CQ efficacy study demonstrated that 4.4% of those tested had CQ-resistant *P. vivax* (*7*). In comparison, the proportion of failures (10.1%) in the current study seems to be relevant; even though most of the *P. vivax* infections (98, 89.9%) were successfully evaluated and adequate clinical and parasitologic responses were obtained. Currently, the FMTAM Manaus Outpatient Clinic is detecting patients from different areas of the city who show parasitologic recurrences after correct treatment within 28 days of the routine clinical follow-up. This observation is an indirect indicator of the possible regional spread of *P. vivax* CQ-resistant strains (unpub. data).

We believe our findings are important and merit the attention of local public health authorities. Considering the possibility of emerging underestimated *P. vivax* CQ resistance in Manaus, we feel it is essential to quickly clarify whether such documented resistance can copromote vivax malaria outbreaks in malaria-endemic areas within the Amazon.

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Franklin Simoes de Santana Filho,* Ana Ruth de Lima Arcanjo,* Yonne Melo Chehuan,* Monica Regina Costa,* Flor Ernestina Martinez-Espinosa,*† Jose Luis Vieira,‡ Maria das Graças Vale Barbosa,*§ Wilson Duarte Alecrim,*¶ and Maria das Graças Costa Alecrim*§¶

*Foundation for Tropical Medicine of Amazonas, Manaus, Amazonas, Brazil; †Foundation for Research Support of Amazonas, Manaus, Amazonas, Brazil; ‡Federal University of Pará, Belém, Pará, Brazil; §Amazonas State University, Manaus, Amazonas, Brazil; and ¶Nilton Lins University, Manaus, Amazonas, Brazil

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Address for correspondence: Franklin Simoes de Santana Filho, The Foundation for Tropical Medicine of Amazonas, Av Pedro Teixeira 25 Planalto, Manaus, Amazonas 69040000, Brazil; email: fsimoes@fmt.am.gov.br

Avian Influenza Risk Perceptions, Laos

To the Editor: After the 2004 outbreak of highly pathogenic avian influenza (HPAI) in poultry in Lao People's Democratic Republic (PDR), the Ministry of Health implemented extensive virologic surveillance (*1*,*2*). Surveillance began in July 2005, and by early 2006, only sporadic cases were found. In July 2006, an outbreak of HPAI was confirmed on 2 chicken farms in Vientiane, the capital city of Lao PDR (*1*,*3*). Most of Laos' ≈20 million chickens are kept on family-owned backyard farms; 3.2 million are on commercial farms (*4*). This production meets 80% of Lao poultry (chicken, duck, goose, quail) needs; imports from neighboring countries, either through legal trade or cross-border smuggling, account for the rest (*3*). Common poultry diseases occur frequently during the cold season, and lack of reporting of poultry deaths is of concern (*4*).

Until February 2007, no human cases of influenza A (H5N1) had been reported in Lao PDR. To learn more about Laotians' knowledge of HPAI and perceptions of their risk, we conducted a cross-sectional survey.

In March–April 2006, participants in 3 settings (Vientiane, urban; Oudomxay, semiurban; Attapeu Province and Hinheub District, both rural) were interviewed in the Lao language by means of a standardized 33-question survey. We recorded information about behavior, poultry handling and keeping practices, and poultry deaths. We used multivariate analysis (Stata, version 8; Stata Corporation, College Station, TX, USA) to analyze the factors associated with behavior changes.

Using a random sampling list of visitors and vendors, we interviewed 461 respondents in 4 Vientiane city markets (Vientiane has 114,793 households and 3,700 registered poultry farms) (*5*). Semiurban respondents were recruited in Oudomxay (40,987 households, 715 poultry farms), an active trading zone near the Chinese border. Rural respondents were recruited from Hinheup District and in Attapeu (19,050 households, 360 poultry farms), near the Vietnam border. Twenty villages were randomly selected, and 10 participants per village were randomly selected for interview. Approval for the investigation was obtained from the health and market authorities. Oral consent for interview was obtained from participants.

A total of 842 participants were interviewed (Table). Differences in occupation and literacy were associated with different study areas. Differences in participant sex and age were also noted because, in the rural areas, interviews took place in the home. A total of 583 (69.3%) participants were female: 302 (65.5%), 139 (68.2%), and 150 (79.3%), in urban, semiurban, and rural areas, respectively; $p = 0.002$, 95% confidence interval 66–72. Mean ages for participants in these areas were 41 (range 40–43), 34 (range 32–36), and 38 (range 37–41) years, respectively; $p<0.001$. Animal breeding was conducted by 50% of families. Daily close exposure to poultry was common (39.6%). Few families owned a henhouse, and no special handling of poultry was reported. Rates of poultry vaccination against common poultry diseases were higher in urban and semiurban areas; veterinary surveillance was low (10.2%).

Overall, 96.9% of respondents had already heard of HPAI, mainly through television. Urban residents ranked it as the most well-known poultry disease, but rural residents ranked it fifth. Less than half of the respondents had some knowledge of the disease signs and symptoms for humans and poultry; 28.4% could describe 1 symptom. Half of the respondents believed that they were not at risk for human avian influenza or that their poultry were not at risk for it. Respondents in urban and semiurban areas knew more about avian influenza than those in rural areas.

During the cold season, poultry deaths were higher in the north (colder) and south than in Vientiane. The poultry mortality rate during the cold season was similar to that of Cambodia *(6)*. Behavior regarding poultry deaths differed between areas. Despite a high rate of poultry deaths, none of the interviewees had notified authorities. Since hearing about HPAI, 67.1%

Table. Avian influenza knowledge, risk perception, and poultry-keeping behavior, Lao People's Democratic Republic*

Characteristic	Urban, n (%)	Semiurban, n (%)	Rural, n (%)	Total, n (%)	p value	95% CI
Total persons interviewed	461	192	189	842		
Illiterate	175 (37.9)	60 (31.2)	181 (95.7)	416 (49.4)	<0.001	47.1–54
Occupation						
Housewife	126 (27.3)	24(12.5)	94 (50)	244 (28.9)	<0.001	32–25.9
Farmer	25 (5.4)	36 (18.75)	75 (40)	136 (16.1)	<0.001	13.7–18.6
Government worker	103 (22.4)	22 (11.5)	3 (1.5)	128 (15.2)	<0.001	12.8–17.6
None	2 (4.3)	0	24 (12.6)	36 (4.2)	<0.001	2.9–5.6
Keep poultry	185 (40.2)	97(50.5)	159 (84.3)	441 (59.4)	<0.001	19 (17–20)
≥1 poultry death, past 2 mo†	58 (31.3)	84 (86.5)	95 (59.7)	239 (54.1)	<0.000	49.5–58.8
Any poultry deaths, past 2 y	95 (51.3)	62 (63.9)	141 (88.6)	298 (65.5)	<0.001	63.2–71.9
Response to dead poultry (n = 399)‡						
Bury dead chickens	105 (56.7)	87 (89.6)	118 (74.2)	310 (70.2)	<0.001	66–74.6
Throw out dead chickens	50 (27.0)	5 (5.1)	9 (5.6)	64 (14.5)	<0.001	11.2–17.8
Eat dead chickens	1 (0.5)	2 (2.0)	7 (4.4)	10 (2.2)	0.06	0.9–3.7
Treat other chickens	0	0	5 (2.6)	5 (0.5)	<0.001	0.07–1.1
Apply lime to backyard	0	8 (1.7)	1 (0.5)	9 (1.0)	<0.001	0.03–1.7
Sell dead chickens	0	1 (1.0)	0	1 (0.1)	0.1	0.00–0.3
Report dead chickens	0	0	0	0	NA	NA
Poultry location						
Henhouse	39 (21.0)	4 (4.4)	7 (4.4)	50 (11.3)	<0.001	8.4–14.3
Inside house	8 (4.3)	1 (1.03)	2 (12.6)	11 (2.4)	0.003	1–3.9
Near house (<5 m)	78 (42.2)	59 (61)	28 (17.7)	165 (37.4)	<0.001	32.9–41.9
Far from house (>5 m)	58 (31.3)	30 (31)	114 (71.7)	202 (45.8)	<0.001	41.2–50.5
Regular poultry vaccination	81 (43.7)	54 (55.6)	19 (11.9)	154 (34.2)	<0.001	30.5–39.4
Information source						
Never heard	8 (1.7)	11 (5.1)	7 (3.7)	26/837 (3.1)	0.02	1.9–4.3
Heard from television	388 (86.4)	158 (87.8)	178 (97.8)	724 (89.2)	<0.001	(86.4–90.8)
Heard from radio	19 (4.2)	12 (6.6)	4 (2.2)	35 (4.3)	0.1	(3.02–5.9)
Read in paper	6 (1.3)	1 (0.5)	0	7 (0.8)	0.003	(0.34–1.8)
Perceive risk for avian influenza						
In Laos	369 (81.6)	110 (60.7)	8 (4.3)	487 (59.6)	<0.001	56.3–63
At home	293 (64.8)	72 (40.0)	5 (2.6)	370 (45.7)	<0.001	41.9–48.8
Unable to describe human disease	116 (25.6)	116 (63.7)	182 (97.5)	414 (50.7)	<0.001	47.3–54.2
Able to describe as lethal for poultry	306 (67.5)	90 (49.7)	2(1.0)	398 (48.7)	<0.0001	45.3–52.2
Behavior change‡	416 (91.8)	125 (69.0)	7 (3.8)	548 (67.1)	<0.0001	63.9–70.4
Stopped eating chicken	328 (72.4)	120 (66.2)	0	448 (54.9)	<0.000	51.5–58.3
Avoided contact	348 (76.8)	60 (33.1)	3 (1.6)	411 (50.3)	<0.000	46.9–53.8
Stopped keeping poultry	335 (73.9)	13 (7.1)	1 (0.5)	349 (42.7)	<0.000	39.4–46.2
Wore mask	338 (74.6)	10 (5.5)	1 (0.5)	349 (42.7)	<0.000	39.4–46.2
Washed hands after contact	100 (22.0)	3 (1.6)	1 (0.5)	104 (12.7)	<0.002	10.5–15
Ate well-cooked chicken	155 (34.2)	3 (1.6)	1 (0.5)	159 (19.4)	<0.000	16.8–22.2

*CI, confidence interval; NA, not applicable.
†Mean nos. of poultry deaths were 15 (range 10–19), 27 (range 22–32), and 15 (range 13–18) for urban, semiurban, and rural areas, respectively. Total mean = 19.3; p<0.0001; 95% CI, 17.0–18.4.
‡95% CIs were 89–94, 62–76, and 1–7 for urban, semiurban, and rural areas, respectively.

respondents, mainly in Vientiane, claimed that they had changed behavior regarding poultry. Multivariate analysis showed the following factors to be associated with behavior change: level of education (p = 0.002), urban living (p<0.001), knowledge of avian influenza risk (p<0.001) and disease (p<0.001), owning poultry (p<0.001), and being a government worker (p<0.001).

This study had limitations but provides new insights on Laotians' knowledge and poultry practices with regard to HPAI. Despite a high level of awareness, populations underestimated the risk, particularly those in rural areas. Most respondents were unaware of appropriate poultry-handling measures to reduce risk (*6*). The claimed changes were higher (more frequent and more substantial) in urban (91.8%) than in rural sites (3.8%, p<0.001), higher than changes made by their counterparts in Thailand (*7*), and confirmed by reports after the 2004 outbreaks (*8*,*9*). These differences between urban and rural areas might be explained not only by participant characteristics but also by a lower extent of the awareness campaign in rural areas.

Failure to report poultry deaths should be addressed and has several possible explanations. Farmers are accustomed to common yearly poultry deaths, which are not reported. In the absence of an official compensation statement, farmers may fear income loss from massive poultry culling.

Our results emphasize the need for more accurate information about transmission risks, notification requirements, safer behavior and practices, and compensation for losses. Focus also needs to be placed on building capacity in the veterinary system (*10*). These issues should be integrated in the Laos National Avian Influenza Control and Pandemic Preparedness Plan (2006–2010).

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Hubert Barennes,*
Bertrand Martinez-Aussel,*
Phengta Vongphrachanh,*†
and Michel Strobel*

*Institut Francophone pour la Médecine Tropicale, Vientiane, Lao PDR; and †Ministry of Health, Vientiane, Lao PDR

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Address for correspondence: Hubert Barennes, Institut Francophone pour la Médecine Tropicale, BP 9516 Vientiane, Lao PDR; email: hubert.barennes@auf.org

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.

Norovirus GII.4 Strains and Outbreaks, Australia

To the Editor: Viral gastroenteritis affects millions of people of all ages worldwide, and some seasonality has been observed in outbreak occurrences (*1*–*3*). During early 2006 in New South Wales (NSW), a marked increase in outbreaks of gastroenteritis occurred (Figure): 155 outbreaks were reported during the first 5 months compared with 88 outbreaks during 2005. During the first 5 months of 2006, the Enteric Pathogens Laboratory–South Eastern Area Laboratory Services (EPL-SEALS) recorded an increase in norovirus in stool samples, detected by using an enzyme immunoassay (IDEIA Norovirus, DakoCytomation, Cambridgeshire, UK). From January through May 2006, the proportion of samples positive for norovirus increased successively: 0/47 (0%), 1/73 (1.4%), 5/169 (3.0%), 8/106 (7.5%), and 93/413 (22.5%). This trend followed the increasing reports of outbreaks made to the NSW Department of Health (Figure). In May, the rate of norovirus detection (22.5%) was significantly greater than that of any other pathogen (Fisher exact test, $p<0.0001$), including intestinal parasites, foodborne bacterial pathogens (*Salmonella, Shigella,* and *Camplylobacter*), and enteric viruses (rotavirus, adenovirus, and astrovirus).

In April 2006, the NSW Department of Health Public Health Realtime Emergency Department Surveillance System (PHREDSS) detected a significant increase in visits for gastroenteritis. This system records cases in real time for each visit to an emergency department from patient demographic information and syndromes diagnosed according to the International Classification of Diseases, version 9, Clinical Modification (ICD-9-CM) (*4*). Information collected came

from a population of >4 million persons, predominantly in the Greater Sydney metropolitan region. During April and May 2006, >8,000 visits for vomiting and diarrhea were recorded in PHREDSS, which is ≈3,000 above the average number of cases for this period for the previous 5 years. During this 8-week period, reports of clinically diagnosed outbreaks in institutional settings also increased; 129 outbreaks affected >3,485 persons. This number of outbreaks is the highest ever reported in the April–May (autumn) period for this region since data for outbreaks (mainly from aged-care facilities, hospitals, childcare centers, and schools) were collected by NSW Department of Health in 2004. Furthermore, laboratories detected norovirus in 37 (28.7%) of 129 outbreaks investigated by public health authorities.

The stool samples examined by EPL-SEALS in May 2006 were from patients treated in public hospitals and nursing homes within the Eastern Sydney and Illawarra regions as well as referred samples from private laboratories serving the Greater Sydney region. Sequencing of a random selection of 15 positive samples representative of the diverse location of case-patients indicated that 10 (66.7%) of 15 were GII.4 genotype. The nucleotide sequence of 266 bp of the N terminus of the capsid gene closely resembled (98%) the sequence of Farmington Hills virus and was 100% identical over the same region to norovirus Rhyl440. The remaining positive strains belonged to GII.3 (4 of 15) and GII.12 (1 of 15). The latter strain, designated Schwerin virus, was previously isolated in outbreaks in Germany during 2000. Two of these sequenced samples were from nursing home outbreaks and typed as GII.3 and GII.4. The association with outbreaks of the remaining 13 sequenced samples is uncertain.

Norovirus epidemics occurred throughout the world in 2002 (*5*) and 2004 (*6*) and were characterized by the large number of persons affected, multiple routes of transmission, and persistence of endemicity despite rigorous control efforts. In Australia from 1997 to 2000, a period of high activity occurred, dominated by the GII.4 epidemic strain designated U.S. 95/96 (*7*). Farmington Hills virus was responsible for subsequent outbreaks in Sydney in 2002 and followed a decline in norovirus outbreaks during 2001. After the 2002 outbreaks in Australia, a period of low norovirus activity persisted before the 2004 emergence of another GII.4 variant, designated Hunter virus (*3*), which predominated in outbreaks in nursing homes and hospitals. More than 400 outbreaks affecting >15,000 persons occurred in NSW during 2004 (*8*). The Hunter virus was subsequently determined to be the etiologic agent in hundreds of outbreaks occurring in Holland (*9*), New Zealand (Gail Greening, pers. comm.), Taiwan, and Japan.

The occurrence of norovirus epidemics in the Australian autumn (March–May) contradicts the perception that the disease is strongly associated with the winter season, when the incidence of respiratory infections increases (*2*). Indeed, other norovirus outbreaks have previously been reported in the summer season (*1*,*3*). The alternating trend of high and low incidence of outbreaks (Figure) may be related to several factors, some of which may have been implicated in these outbreaks. Such factors include development of herd immunity to the dominating strain, which is short-term; emergence of an epidemic strain with no herd immunity; increased genomic variation due to point mutation or recombination (*10*); or other mechanisms.

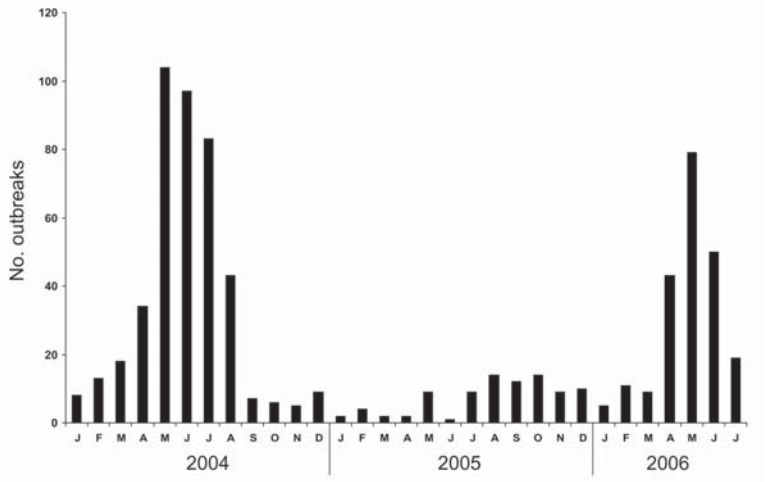

Figure. Number of outbreaks reported to the New South Wales Department of Health, January 2004–July 2006.

Elise T.-V. Tu,* Thanh Nguyen,† Phoebe Lee,† Rowena A. Bull,* Jennie Musto,‡ Grant Hansman,§ Peter A. White,*† William D. Rawlinson,*† and Christopher J. McIver*†

*University of New South Wales, Sydney, New South Wales, Australia; †Prince of Wales Hospital, Sydney, New South Wales, Australia; ‡New South Wales Department of Health, Sydney, New South Wales, Australia; and §National Institute of Infectious Diseases, Tokyo, Japan

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Address for correspondence: Christopher J. McIver, Department of Microbiology, South Eastern Area Laboratory Service, Prince of Wales Hospital, High St, Randwick, New South Wales 2031, Australia; email: christopher.mciver@sesiahs.health.nsw.gov.au

Echinostoma malayanum Infection, the Philippines

To the Editor: In 2002, the Department of Health-Provincial Health Team (DOH-PHT) of Surigao del Norte reported 102 cases of fasciolid-like infections in the municipality of Santa Monica, Siargao Island, the Philippines. The reports were based on characteristic large operculate eggs having been found in routine stool examinations conducted during schistosomiasis surveys. *Fasciola hepatica* infection was the initial diagnosis considered. In 2005, a collaborative team from DOH-PHT, the National Institutes of Health, the University of the Philippines Manila, and the Local Government Unit/Rural Health Unit conducted a field investigation to determine the cumulative prevalence of intestinal helminthic infections in adult patients whose conditions had been previously diagnosed as fasciolid-like infection and to determine the causative species of trematode.

The study group consisted of 70 adult patients from the *barangay* (local government unit of 50–100 families) of Libertad, for whom fasciolid infections had been noted on previous surveys. Researchers confirmed infections and collected adult trematodes for species identification. All patients underwent bowel preparation with bisacodyl (Dulcolax) 10-mg tablets (2 tablets taken immediately after a meal on night before deworming), followed by praziquantel (25 mg/kg in 2 doses 4 h apart), and 30-g magnesium sulfate granules, dissolved in 1 cup of milk, given 1 h after the second dose of praziquantel. Stools were processed by using the Kato-Katz method (*1*) and examined microscopically by medical technologists from the Diagnostic Parasitology Laboratory, College of Public Health, University of the Philippines Manila, for intestinal helminth ova. In addition, a clinical history was taken and a complete physical examination was conducted for each patient volunteer after stool submission. Eating preferences and habits were specifically noted.

The research was approved by the Department of Health Center for Health Development of the Caraga region. Informed consent was obtained before procedures were done and treatment was given to infected patients.

Cumulative prevalence for soil-transmitted helminth infections among the 70 patients was 51.4%. Prevalence according to species was *Trichuris* spp. 42.9%, *Ascaris* spp. 17.1%, and hookworm spp. 1.4%. *Schistosoma japonicum* infection rate was 10%. Stool samples from 8 (11.4%) patients had large (120–130 μm × 80–90 μm), brownish, operculated eggs; 3 had a total of 13 adult flukes. Microscopy showed small leaflike flukes 8–9 mm long and 2.5–3.5 mm wide. After the organisms were processed and stained with aceto-carmine and fast green stains, diagnostic features of *Echinostoma malayanum* (Leiper 1911) were noted. Adult trematodes were within known species size range (5–10 mm × 2.5–3.0 mm) and had elongated bodies and bluntly rounded ends. The ventral sucker (acetabulum) was prominent and larger than the anterior oral sucker. Paired testes were deeply branched and positioned high in the posterior half of the body, extending above the midplane with a single anterior globular ovary. The uterus was entirely anterior to the ovary, and vitellaria (glands) were abundant along both lateral portions of the worm, ending just posterior to the esophagus. The oral sucker had a horseshoe-shaped anterior collar with 43 circumoral spines, which differentiates this species from *E. ilocanum* (49–51 collar spines), another trematode species endemic to the Philippines.

In terms of eating habits, patients reported that fish were commonly eaten raw, after being dipped in a salt

and vinegar mixture, locally known as *kinilaw.* Other methods of fish preparation were *tinola* (boiled), *ginataan* (stewed in coconut milk), and *sinugba* (charcoal-grilled). All echinostome-infected patients had a history of having eaten snails, *kuhol* and *kiambu-ay*, prepared raw with coconut milk and lime juice (*kinilaw)*, especially when found in greater abundance during the rainy season.

Human echinostome infection results from ingestion of metacercariae that encyst in secondary intermediate hosts, usually freshwater snails, tadpoles, or fish. *E. malayanum* uses various species of gastropod mollusks for primary and secondary intermediate developmental stages (*2*–*5*). Certain species of fish may also serve as secondary intermediate hosts (*2*). Several mollusks that may serve as primary and secondary intermediate hosts have been identified in the Philippines, including *Lymnaea (Bullastra) cumingiana, Radix quadrasi*, and *Physastra hungerfordiana* for *E. malayanum*, and *Pila luzonica* for *E. ilocanum* (*6*,*7*).

To our knowledge, this is the first report of *E. malayanum* infections in the southern Philippines. Local eating habits are a strong factor in echinostome infections. The general lack of awareness by health staff and the community was a big factor in the poor identification of the disease. Clinical and laboratory staff and healthcare providers need training about echinostome infections and other intestinal foodborne trematode infections. Similar environmental, sanitary, and eating practices in the region suggest that the same parasitoses should be considered to be widespread in the area. Redirecting vital resources of the local health and government units of the Caraga region to the periphery and building local capacity will help empower authorities to provide public health services in rural areas, strengthen public health programs, and further develop public health infrastructure. Moreover, a successful control program against chronic intestinal parasitoses could serve as a paradigm for local health system development of effective control measures for other endemic diseases.

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Vicente Y. Belizario,*†
Giovanni G. Geronilla,*†
Marilyn Benedith M. Anastacio,*†
Winifreda U. de Leon,†
Adriano P. Suba-an,‡
Arlene C. Sebastian,§
and Michael J. Bangs¶

*National Institutes of Health, Manila, the Philippines; †University of the Philippines Manila, Manila, the Philippines; ‡Health Development, Caraga Region, Butuan City, the Philippines; §Rural Health Unit, Santa Monica, the Philippines; and ¶Navy Region Northwest, Silverdale, Washington, USA

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Address for correspondence: Vicente Y. Belizario, Jr., National Institutes of Health and College of Public Health, University of the Philippines Manila, Pedro Gil, Ermita, Manila, the Philippines; email: vbelizar@yahoo.com

Zoonotic Pathogens in *Ixodes scapularis*, Michigan

To the Editor: *Ixodes scapularis*, the black-legged tick, is the predominant vector of reportable human vectorborne disease in the United States. It transmits agents that cause Lyme borreliosis, human anaplasmosis, and human babesiosis. *I. scapularis*–borne disease is becoming more frequent as this tick expands its range from tick-endemic foci in the northeastern and upper midwestern United States.

Despite Michigan's proximity to large tick-endemic areas (Wisconsin and Minnesota to the west and Indiana to the south), active and passive surveillance data indicated that the only populations of *I. scapularis* established in the state before 2002 were in Menominee County in the Upper Peninsula (*1*,*2*). However, wildlife sampling and tick dragging in 2002–2003 suggested that *I. scapularis* had begun to invade southwestern Michigan (*3*), with nearby populations in northwestern Indiana (*4*) as the putative source.

Because we suspected these invading ticks were bringing zoonotic pathogens into southwestern Michigan, we assessed pathogen prevalence within the state's invading and endemic *I. scapularis* populations. Over a 1.5-week period in April–May 2006, we collected adult *I. scapularis* by drag sampling at 3 recently invaded sites in southwestern Michigan and 2 tick-endemic sites in Menominee County. We targeted adult *I. scapularis* in the spring because this life stage has had 2 chances of becoming infected and because the adult questing peak in Michigan is greater in spring than fall (*2*,*3*).

All collected ticks were bisected aseptically, and total DNA was extracted from half after overnight lysis (DNeasy Tissue Kit; QIAGEN, Valencia, CA, USA). We used 3 PCRs to assay for *Borrelia burgdorferi*, *B. lonestari*, and *B. miyamotoi* (*5*); *Anaplasma phagocytophilum* (*6*); and *Babesia* spp., including *Babesia microti* and *Babesia odocoilei* (*7*). *Borrelia*-positive and *Babesia*-positive amplicons were purified and sequenced for species identification.

Tick densities were highest overall at tick-endemic Menominee County sites; in southwestern Michigan, they were highest at those sites closest to the putative source of the Indiana invasion. We collected 28 adult and 1 nymphal *I. scapularis* and 2 adult *Dermacentor variabilis* from tick-endemic sites. Of the adult *I. scapularis*, 17 (60.7%) were positive for *B. burgdorferi*, 4 (14.3%) were positive for *A. phagocytophilum*, and 2 (7.1%) were positive for *Babesia odocoilei* (Table). We also collected 91 adult and 10 nymphal *I. scapularis* and 5 adult *D. variabilis* from newly invaded sites. Of the adult *I. scapularis*, 43 (47.3%) were positive for *B. burgdorferi*, 1 (1.1%) was positive for *A. phagocytophilum*, and 4 (4.4%) were positive for *Babesia odocoilei*. All 4 *Babesia odocoilei*–positive ticks were co-infected with *B. burgdorferi* (this rate of co-infection was significantly greater than random expectation; p = 0.046, by Fisher exact test).

Within the tick-endemic area, comparison with prior survey data (*8*) indicated that the *B. burgdorferi* infection rate in adult ticks increased from 31.3% in 1992 to 60.7% in the present survey (p<0.001, by Fisher exact test). A similar increasing trend was evident in the invasion area, where prevalence increased from 37.0% in 2002–2003 (at a collection site 5 km south of our southernmost site; [*3*]) to 47.3% in 2006. This latter trend was only marginally statistically significant due to small sample size and the short period between surveys (p = 0.046, by 1-tailed Fisher exact test).

B. burgdorferi infection in *I. scapularis* has been reported in Michigan (*1*–*3*,*8*). To our knowledge, ours is the first report of *A. phagocytophilum* and *Babesia odocoilei* in ticks in Michigan; they are present in both the endemic and recently invaded populations. Similar infection rates for these pathogens have been reported in *I. scapularis* from Indiana (*9*). *B. burgdorferi* and *A. phagocytophilum* are human pathogens; *Babesia odocoilei*, an intraerythrocytic protozoan parasite maintained in transmission cycles in white-tailed deer, is not known to be pathogenic to humans (*7*). Several other *Borrelia* and *Babesia* species (i.e., *B. lonestari*, *B. miyamotoi*-like spirochetes, and *Babesia microti*) from US ticks were not detected in our sample. *I. scapularis* nymphs, which are epidemiologically important (*10*), were not the focus of our sampling. However, several were collected, including some infected with *B. burgdorferi*, *A. phagocytophilum*, or both (Table).

These data imply a risk for Lyme borreliosis and human anaplasmosis in areas endemic for and recently invaded by *I. scapularis*. For example, Lyme disease incidence in the tick-endemic zone has increased significantly over the past 10 years (from 0.33 to 1.53 cases per 10,000 persons during 1997–2006; $r^2 = 0.56$, p = 0.01). Incidence in the invasion zone has

Table. Prevalence of 3 pathogens in *Ixodes scapularis* ticks from 2 Michigan field sites, spring 2006*

			No. ticks infected or co-infected (%)				
Site	Life stage	No. *Ix. scapularis*	*Borrelia burgdorferi*	*Anaplasma phagocytophilum*	*Babesia odocoilei*	*B. burgdorferi* and *A. phagocytophilum*	*B. burgdorferi* and *B. odocoilei*
E-1	A	16	9 (56.3)	1 (6.3)	1 (6.3)	1 (6.3)	0
E-2	A	12	8 (66.7)	3 (25.0)	1 (8.3)	1 (8.3)	1 (8.3)
	N	1	1 (100.0)	0	0	0	0
I-1	A	4	2 (50.0)	0	0	0	0
	N	2	0	1 (50.0)	0	0	0
I-2	A	18	9 (50.0)	0	1 (5.6)	0	1 (5.6)
	N	8	2 (25.0)	1 (12.5)	0	1 (12.5)	0
I-3	A	69	32 (46.4)	1 (1.4)	3 (4.3)	0	3 (4.3)
All endemic sites	A	28	17 (60.7)	4 (14.3)	2 (7.1)	2† (7.1)	1† (3.6)
	N	1	1 (100)	0	0	0	0
All invaded sites	A	91	43 (47.3)	1 (1.1)	4 (4.4)	0	4‡ (4.4)
	N	10	2 (20.0)	2 (20.0)	0	1† (10.0)	0

*E, endemic site; A, adult: N, nymph; I, invaded site.
†Nonsignificant level of co-infection; p = 0.378–0.640, by Fisher exact test.
‡Significant level of co-infection; p = 0.046, by Fisher exact test.

been much lower (mean 0.03 cases per 10,000 persons over same period) but appears to be increasing. Further increases in tick population size, infection, and co-infection can be expected as the invasion continues (*9*). Thus, medical practitioners in southwestern Michigan should be aware of the changing increasing risk for tickborne diseases and consider disease resulting from these pathogens during diagnosis.

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Sarah A. Hamer,*
Pamela L. Roy,*
Graham J. Hickling,†
Edward D. Walker,*
Erik S. Foster,‡
Christina C. Barber,*
and Jean I. Tsao*

*Michigan State University, East Lansing, Michigan, USA; †University of Tennessee, Knoxville, Tennessee, USA; and ‡Michigan Department of Community Health, East Lansing, Michigan, USA

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Address for correspondence: Sarah A. Hamer, Department of Fisheries and Wildlife, Michigan State University, 13 Natural Resources Bldg, East Lansing, MI 48824, USA; email: hamer@msu.edu

The Ghost Map

Steven Johnson

Riverhead Books, New York, New York, USA, 2006
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Widely viewed as the "father of contemporary epidemiology," Dr. John Snow is among the most famous of public health figures (*1*). His "grand experiment" in 1854 (comparing cholera deaths in South London households that had consumed contaminated water with those that had not consumed contaminated water) is often considered a classic (*2*), but the Broad Street pump outbreak is perhaps the more famous historical account and is the subject of Steven Johnson's new book, The Ghost Map.

Dr. Snow wrote: "The most terrible outbreak of cholera which ever occurred in this kingdom, is probably that which took place in Broad Street, Golden Square, and the adjoining streets, a few weeks ago. Within two hundred and fifty yards of the spot where Cambridge Street joins Broad Street, there were upwards of five hundred fatal attacks of cholera in ten days. The mortality in this limited area probably equals any that was ever caused in this country, even by the plague; and it was much more sudden, as the greater number of cases terminated in a few hours" (*2*).

Although this 1854 outbreak is mentioned in many public health and epidemiology texts, the focus is usually on data gathering and presentation, and the actions taken to address the outbreak. What is not often conveyed is the social environment of the times or the role of Reverend Henry Whitehead in dealing with this fearsome outbreak. Steven Johnson addresses these omissions in The Ghost Map and brings forth aspects of John Snow's life in an insightful, riveting manner.

Johnson's opening sentences provide a sense of what is to come: "It is August 1854, and London is a city of scavengers. Just the names alone read now like some kind of exotic zoological catalogue: bone pickers, rag-gatherers, pure-finders, dredgermen, mud-larks, sewer-hunters, dustmen, night-soil men, bunters, toshers and shoremen." He goes on to describe their roles in Victorian London and provides the reader with an intimate feel for local life, notably the travails of getting water and disposing of sewage. Along the way, the reader meets a local clergyman, Henry Whitehead, whose affable nature is in contrast to that of the more stoic John Snow. Yet, these 2 men of varied backgrounds become entwined by the Broad Street outbreak, using their complementary skills to help solve an epidemiologic mystery.

The Ghost Map scarcely mentions the contributions of William Farr and other notables of the times. Instead, being a novel rather than a treatise, the book attempts to breathe life into a few seminal characters. Johnson is an excellent writer. His words evoke strong images that revolve in the mind. He uses London and Snow's classic map of the 1854 outbreak as the focal points of his story, along with the removal of the Broad Street pump handle and the discovery of the probable index case. This is a good read, highly recommended for those open to the contributions of our forebearers in public health and the link of 19th-century London to modern day urban life.

Ralph R. Frerichs*

*University of California at Los Angeles, Los Angeles, California, USA

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Address for correspondence: Ralph R. Frerichs, Department of Epidemiology, 650 Charles E. Young Circle Dr South, Los Angeles, CA 90095-1772, USA; email: frerichs@ucla.edu

Rowing on the Schuylkill, Damming on the Yangtze

Polyxeni Potter*

Thomas Eakins (1844–1916)
John Biglin in a Single Scull (1874)
Oil on canvas (61.9 cm × 40.6 cm)
Yale University Art Gallery. Whitney Collections of Sporting Art, given in memory of Harry Payne Whitney, BA 1894 and Payne Whitney, BA 1898, by Francis P. Garvan, BA 1897, MA (Hon.) 1922

"Should the he-painters draw the horses and bulls, and the she-painters…the mares and cows?" asked Thomas Eakins, when critics derided his use of nude models in the presence of women art students (*1*). Eakins made no bones about his teaching practices and no compromises in his pursuit of artistic excellence. So he was viewed as radical and irascible, and although his art was widely discussed and exhibited, he did not see commercial success during his lifetime—he only sold 30 paintings.

A man ahead of his time, Eakins was born in Philadelphia, the son of a calligrapher, who nurtured his artistic talent and taught him the value of exacting detail. "I was born July 25, 1844. My father's father was from the north of Ireland of the Scotch Irish. On my mother's side my blood is English and Hollandish. I was a pupil of Gérôme (also of [portrait painter] Bonnat and Dumont, [the] sculptor). I have taught in life classes and lectured on anatomy continuously since 1873. I have painted many pictures and done a little sculpture….I believe my life is all in my work," he wrote (*2*).

He studied drawing at the Pennsylvania Academy of Fine Arts and anatomy at Jefferson Medical College, traveled to Paris to attend the École des Beaux-Arts, and near the end of his studies, visited Spain "to see the pictures" (*2*). Despite his studies in Paris, he was most influenced by 17th-century Dutch and Spanish painters, particularly Diego Velázquez and Jusepe de Ribera. He lived the rest of his life in his beloved Philadelphia, following his own advice on achieving greatness: "remain in America to peer deeper into the heart of American life" (*3*).

Philadelphia and the Schuylkill River, which runs through it, held a special fascination for Eakins. He delighted in sailing, swimming, rowing, and all manner of outdoor activity before and after his travels abroad. Rowing, already a popular sport, attracted large crowds in the 1850s, when several rowing clubs formed the Schuylkill Navy, now the oldest amateur athletic governing body in the United States. As Eakins began his career and sought subjects from his immediate surroundings, he got caught up in the excitement of the sport, becoming one of the first artists to portray rowers in action. Sometimes he placed himself in the pictures and inscribed his name on the scull.

His painting of the human form, encouraged during his studies from nude models in Paris, was all but stifled by local culture, but the semi-nude athletic figure was socially acceptable. He produced nearly 30 rowing pictures from 1871 to 1874, at first painting his childhood friend Max Schmitt and later the Biglin brothers, a pair of celebrity rowers from New York. Still, reviews in the Philadelphia Inquirer were not glowing, "The artist, in dealing so boldly and broadly with the commonplace in nature, is working upon well-supported theories, and, despite somewhat scattered effect, gives promise of a conspicuous future" (*4*).

As teacher and later director of the Pennsylvania Academy of the Fine Arts, he introduced anatomy, dissection, and scientific perspective into the curriculum, revolutionizing art instruction. But he also scandalized school authorities with the use of nude models and was forced to resign in 1886. He continued to paint. "I

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

will never have to give up painting, for even now I could paint heads good enough to make a living anywhere in America" (*5*). Later he was recognized for his formidable talent and was elected to the National Academy of Design. Provocative behavior though continued to damage his reputation, "My honors are misunderstanding, persecution, and neglect" (*5*).

Eakins' approach to painting relied on close observation. He rejected embellishment and sentimentality and was the only artist, his friend Walt Whitman said, "who could resist the temptation to see what [he] think[s] ought to be rather than what is." During the latter part of his career, he focused on portraiture: studies of relatives, friends, and persons accomplished in the sciences and other disciplines. The subject of a fine portrait in 1888, Whitman called Eakins "not so much a painter, as a force" (*6*). Unlike his contemporaries James McNeill Whistler, John Singer Sargent, and William Merritt Chase, popular society portraitists, Eakins painted his subjects with uncompromising realism and meticulous precision, which lent them a somber, aged, sometimes unflattering, aspect. While Chase's studio was an atelier, Eakins joked, his own was a workshop.

One of his portraits, The Gross Clinic, widely acclaimed as the greatest American painting of the 19th century, depicts surgeon Samuel D. Gross performing surgery, instructing students, and training assistants to remove bone from the leg of an anesthetized patient. The portrait scandalized Victorian society. "It is a picture that even strong men find it difficult to look at long, if they can look at it at all"; wrote The New York Tribune, "and as for people with nerves and stomachs, the scene is so real that they might as well go to a dissecting room and have done with it" (*7*).

In John Biglin in a Single Scull, on this month's cover, Eakins brought to bear his personal experience as rower and knowledge of the muscles involved. John Biglin, a "physical specimen…about as near perfect as can be found," dominated the rowing scene in the 1860s and 70s (*8*). Sculpted as in relief, the figure is focused and intense, muscles terse, shoulders rounded. The composition is economical and accurate, from the sports hero's facial features to the slightly worn wooden thole pin that held the oar in place for rowing. John Biglin, the quintessential outdoorsman equivalent of Samuel Gross the heroic physician!

Water activities continue on the Schuylkill and elsewhere, and the excitement of rowing remains undiminished as does the enjoyment of art. Our close relationship with water, far more complex than Eakins' luminous river would suggest, has only become closer with better understanding of biology. In the 1850s, while rowing was becoming popular in Philadelphia, John Snow, the "father of epidemiology," was investigating the water supply and sewage disposal in South London and finding that cholera is waterborne (*9*).

More than a hundred years later, diarrhea is the leading cause of childhood deaths in places that must rely on drinking water contaminated with pathogens (*10*). Invasive water organisms are spreading fast around the globe, damaging agriculture (*11*). And human activities, such as the Three Gorges Dam construction across the Yangtze River in People's Republic of China, are threatening changes in ecology and setbacks in schistosomiasis control (*12*).

Water quality has environmental and social components. It is like good painting, which Eakins believed, extends beyond the geometry of landscape and the refraction of light on the waves to provide full understanding. Or, as he put it, "You can see what o'clock it is afternoon or morning if it's hot or cold winter or summer and what kind of people are there and what they are doing and why they are doing it" (*13*).

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the July issue for the following topics:

Brazilian Vaccinia Viruses and their Origins

Water Management and Schistosomiasis, Dongting Lake Region, China

Thottapalayam Hantavirus

Antimicrobial Drugs and Community-Acquired MRSA

Klebsiella pneumoniae Bloodstream Infections and Virulence Characteristics

Antiretroviral Therapy during Tuberculosis Treatment of HIV-Infected Patients

Ongoing Genome Reduction in *Mycobacterium ulcerans*

Rift Valley Fever Outbreak in West Africa and East-Central African Virus Lineage

Linezolid-Resistant Enterococci

Person-to-Person Transmission of Nipah Virus

Effects of Internal Border Control on Spread of Pandemic Influenza

Emergency Authorization for Product Use in Civilian and Military Emergencies

Influenza Pandemics in Singapore

Live Poultry Exposures, Hong Kong and Hanoi

Cutaneous Leishmaniasis, Sri Lanka

Norovirus in a Captive Lion (*Panthera leo*)

Complete list of articles in the July issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

July 16–18, 2007
Public Health Congress
Mandarin Oriental
Washington, DC, USA
http://www.publichealthcongress.com

July 29–31, 2007
Immunobiology of Influenza Virus Infection: Approaches for an Emerging Zoonotic Disease
University of Georgia Center for Continuing Education Conference Center and Hotel
Athens, GA, USA
http://www.virus-eid.org

July 29–August 1, 2007
17th ISSTDR Meeting | 10th IUSTI World Congress
Seattle, WA, USA
http://www.isstdr.org

August 6–17, 2007
10th International Dengue Course
Havana, Cuba
email: lupe@ipk.sld.cu
http://www.ipk.sld.cu/cursos/dengue2007/index.htm

August 9–11, 2007
Symposium "25 Years of Experience Struggling against Dengue"
Havana, Cuba
email: lupe@ipk.sld.cu
http://www.ipk.sld.cu/cursos/dengue2007/simpoen.htm

September 17–20, 2007
47th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
Chicago, IL, USA
http://www.icaac.org

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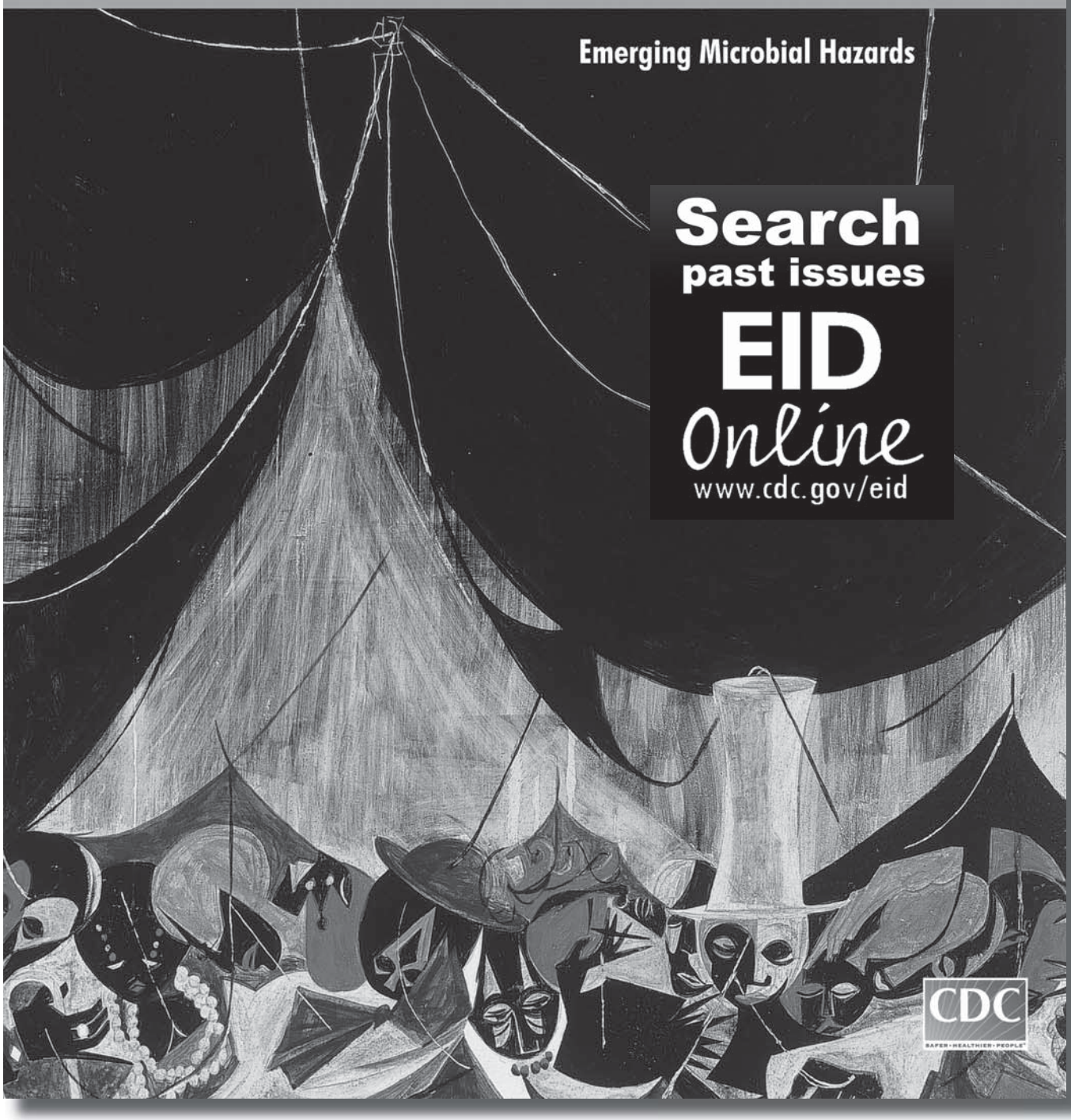

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

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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 of first author. 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.

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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 of first author—both authors if only 2. 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 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 of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

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