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On the Cover

Rembrandt van Rijn (1606–1669)
Aristotle with a Bust of Homer
(1653) Oil on canvas
(143.5 cm × 136.5 cm).
The Metropolitan Museum of Art,
New York, NY

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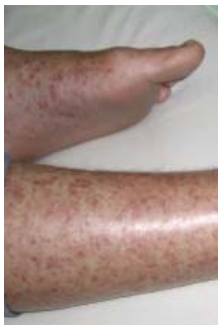
Research



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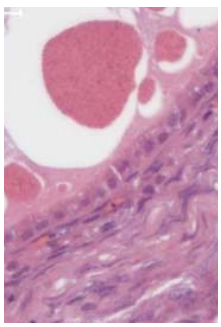

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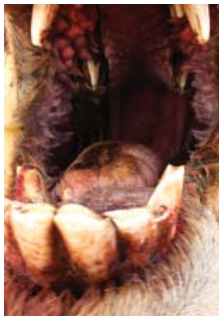

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Global Spread of Carbapenemase-producing *Enterobacteriaceae*

Patrice Nordmann, Thierry Naas, and Laurent Poirel

Carbapenemases increasingly have been reported in *Enterobacteriaceae* in the past 10 years. *Klebsiella pneumoniae* carbapenemases have been reported in the United States and then worldwide, with a marked endemicity at least in the United States and Greece. Metallo-enzymes (Verona integron–encoded metallo-β-lactamase, IMP) also have been reported worldwide, with a higher prevalence in southern Europe and Asia. Carbapenemases of the oxacillinase-48 type have been identified mostly in Mediterranean and European countries and in India. Recent identification of New Delhi metallo-β-lactamase-1 producers, originally in the United Kingdom, India, and Pakistan and now worldwide, is worrisome. Detection of infected patients and carriers with carbapenemase producers is necessary for prevention of their spread. Identification of the carbapenemase genes relies mostly on molecular techniques, whereas detection of carriers is possible by using screening culture media. This strategy may help prevent development of nosocomial outbreaks caused by carbapenemase producers, particularly *K. pneumoniae*.

Enterobacteriaceae are inhabitants of the intestinal flora and are among the most common human pathogens, causing infections such as cystitis and pyelonephritis with fever, septicemia, pneumonia, peritonitis, meningitis, and device-associated infections. *Enterobacteriaceae* are the source of community- and hospital-acquired infections. They have the propensity to spread easily between humans (hand carriage, contaminated food and water) and to acquire genetic material through horizontal gene transfer, mediated mostly by plasmids and transposons.

Since 2000, spread of community-acquired enterobacterial isolates (*Escherichia coli*) that produce extended-spectrum β-lactamases (ESBLs) capable of hydrolyzing almost all cephalosporins except carbapenems has been reported worldwide (*1*). It is therefore mandatory to maintain the clinical efficacy of carbapenems (imipenem, ertapenem, meropenem, doripenem), which have become antimicrobial drugs of last resort. These agents are crucial for preventing and treating life-threatening nosocomial infections, which are often associated with techniques developed in modern medicine (transplantation, hospitalization in an intensive care unit, highly technical surgery).

Carbapenem-resistant *Enterobacteriaceae* have been reported worldwide as a consequence largely of acquisition of carbapenemase genes (*2*). The first carbapenemase producer in *Enterobacteriaceae* (NmcA) was identified in 1993 (*3*). Since then, a large variety of carbapenemases has been identified in *Enterobacteriaceae* belonging to 3 classes of β-lactamases: the Ambler class A, B, and D β-lactamases (*2*). In addition, rare chromosome-encoded cephalosporinases (Ambler class C) produced by *Enterobacteriaceae* may possess slight extended activity toward carbapenems, but their clinical role remains unknown (*2*,*4*).

Class A Carbapenemases

A variety of class A carbapenemases have been described; some are chromosome encoded (NmcA, Sme, IMI-1, SFC-1), and others are plasmid encoded (*Klebsiella pneumoniae* carbapenemases [KPC], IMI-2, GES, derivatives), but all effectively hydrolyze carbapenems and are partially inhibited by clavulanic acid (*2*). KPCs are the most clinically common enzymes in this group. The first KPC producer (KPC-2 in *K. pneumoniae*) was identified in 1996 in the eastern United States (*5*).Within a few years, KPC producers had spread globally and have been described across the contiguous United States (still mostly in eastern coast states) and, in particular, in Puerto Rico, Colombia, Greece, Israel, and the People's Republic of China (*6*,*7*) (Figure 1). Outbreaks of KPC producers also have been reported in many European countries and in South America (*6*,*7*) (Figure 1).

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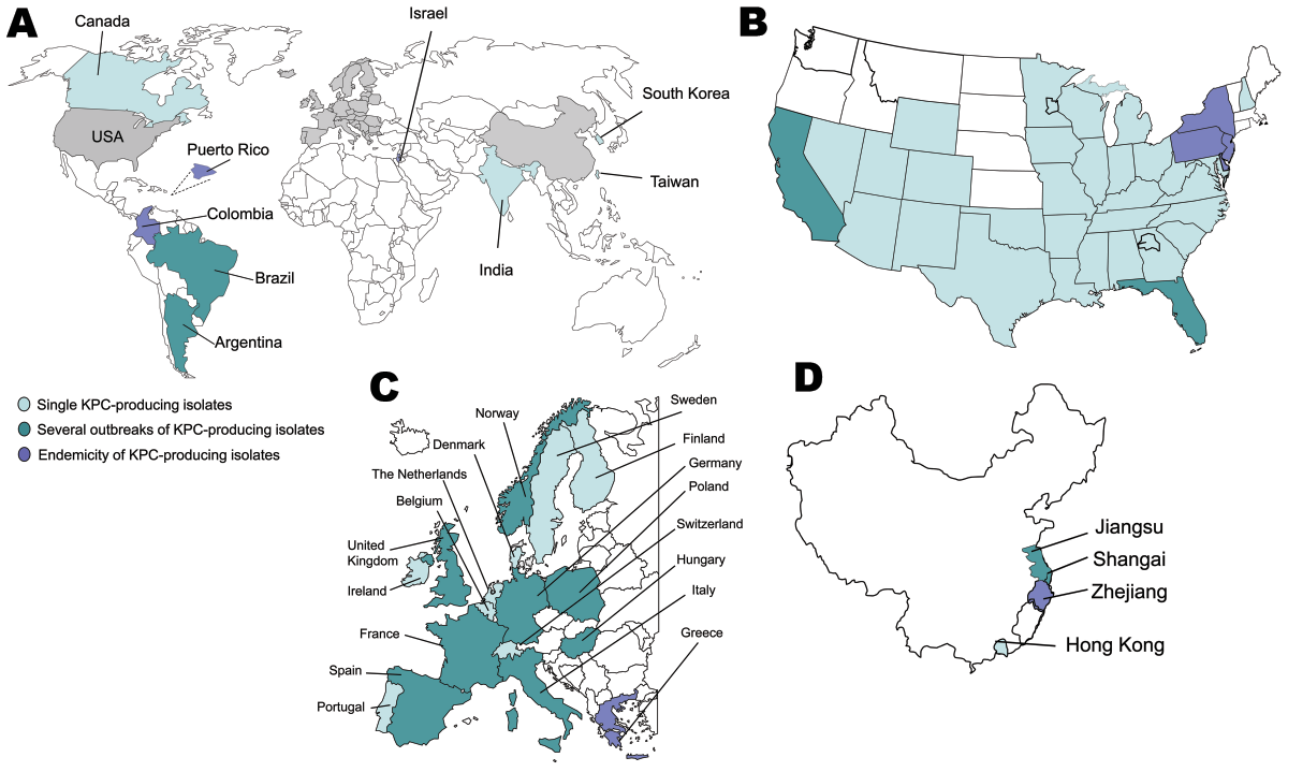

Figure 1. A) Worldwide geographic distribution of *Klebsiella pneumoniae* carbapenemase (KPC) producers. Gray shading indicates regions shown separately: B) distribution in the United States; C) distribution in Europe; D) distribution in China.

KPC producers have been reported, mostly from nosocomial *K. pneumoniae* isolates and to a much lesser extent from *E. coli* (especially in Israel) and from other enterobacterial species (*6*). A single *K. pneumoniae* clone (sequence type [ST]-258) was identified extensively worldwide, indicating that it may have contributed to the spread of the bla_{KPC} genes (*8*).Within a given geographic location, several KPC clones are disseminating that differ by multilocus sequence type; additional β-lactamase content; and by size, number, and structure of plasmids, but the bla_{KPC} genes are associated with a single genetic element (transposon Tn*4401*) (*8*). Although community-acquired KPC producers have been reported, they are rare, with the exception of isolates from Israel a few years ago (*6*).The level of resistance to carbapenems of KPC producers may vary markedly; ertapenem is the carbapenem that has the lowest activity (*5*–*7*), (Table 1). KPC producers are usually multidrug resistant (especially to all β-lactams), and therapeutic options for treating KPC-related infections remain limited (*6*) (Figure 2, panel A). Death rates attributed to infections with KPC producers are high (≥50%) (*9*–*11*).

Class B Metallo-β-Lactamases

Class B metallo-β-lactamases (MBLs) are mostly of the Verona integron–encoded metallo-β-lactamase (VIM) and IMP types and, more recently, of the New Delhi metallo-β-lactamase-1 (NDM-1) type (*2*,*12*).The first acquired MBL, IMP-1, was reported in *Serratia marcescens* in Japan in 1991 (*13*). Since then, MBLs have been described worldwide (*2*,*12*) (Figure 3). Endemicity of VIM- and IMP-type enzymes has been reported in Greece, Taiwan, and Japan (*2*,*12*), although outbreaks and single reports of VIM and IMP producers have been reported in many other countries (Figure 3). These enzymes hydrolyze all β-lactams except aztreonam (*12*).Their activity is inhibited by EDTA but not by clavulanic acid (*12*). Most MBL producers are hospital acquired and multidrug-resistant *K. pneumoniae* (*2*,*12*). Resistance levels to carbapenems of MBL producers may vary (Table 1). Death rates associated with MBL producers range from 18% to 67% (*14*).

Discovered in 2008 in Sweden from an Indian patient hospitalized previously in New Delhi (*15*), NDM-

Table 1. MIC range of carbapenems for *Enterobacteriaceae* that produce several types of carbapenemases*

	MIC, mg/L		
Carbapenemase	Imipenem	Meropenem	Ertapenem
KPC	0.5–>64	1–>64	0.5–>64
Metallo β-lactamases†	0.5–>64	0.25–>64	0.5–>64
OXA-48 type	1–>64	0.5–>64	0.25–>64

*KPC, *Klebsiella pneumoniae* carbapenemase; OXA-48, oxacillinase-48.
†Including New Delhi metallo-β-lactamase-1.

1–positive *Enterobacteriaceae* are now the focus of worldwide attention (*15*–*17*). Since mid-August 2010, NDM-1 producers have been identified on all continents except in Central and South America with, in most of the cases, a direct link with the Indian subcontinent (*17*) (Figure 4). Few cases have been reported from the United States and Canada (*17*). Recent findings suggest that the Balkan states and the Middle East may act as secondary reservoirs of NDM-1 producers (*17*) (Figure 4).

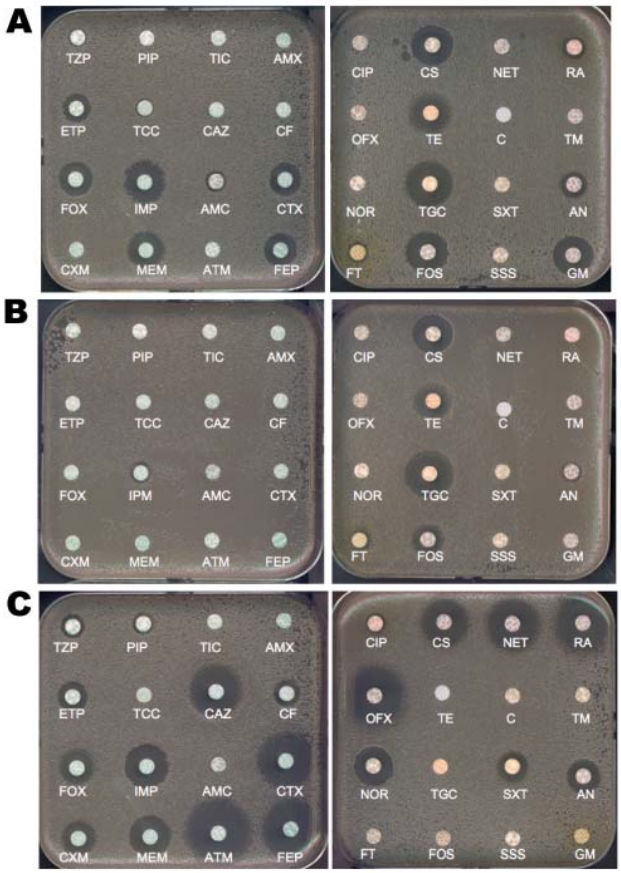

Figure 2. Disk diffusion antibacterial drug susceptibility testing of A) *Klebsiella pneumoniae* carbapenemase-2 (KPC-2)–, B) New Delhi metallo-β-lactamase-1 (NDM-1)–, and C) oxacillinase-48 (OXA-48)–producing K. pneumoniae clinical isolates. Clinical isolates producing KPC-2 and OXA-48 do not co-produce other extended-spectrum β-lactamase, but the isolate producing NDM-1 co-produces the extended-spectrum β-lactamase CTX-M-15. Wild-type susceptibility to β-lactams of *K. pneumoniae* includes resistance to amoxicillin, ticarcillin, and reduced susceptibility to piperacillin and cefalotin (data not shown).TZP, piperacillin/tazobactam; PIP, piperacillin; TIC, ticarcillin; AMX, amoxicillin; ETP, ertapenem; TCC, ticarcillin/clavulanic acid; CAZ, ceftazidime; CF, cefalotin; FOX, cefoxitin; IMP, imipenem; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CXM, cefuroxime; MEM, meropenem; ATM, aztreonam; FEP, cefepime; CIP, ciprofloxacin; CS, colistin; NET, netilmicin; RA, rifampin; OFX, ofloxacin; TE, tetracycline; C, chloramphenicol; TM, tobramycin; NOR, norfloxacin; TGC, tigecycline; SXT, sulfamethoxazole/trimethoprim; AN, amikacin; FT, nitrofurantoin; FOS, fosfomycin; SSS, sulfamethoxazole; GM gentamicin.

In contrast to several other carbapenemase genes, the $bla_{\text{NDM-1}}$ gene is not associated with a single clone but rather with nonclonally related isolates and species (*16*,*17*). It has been identified mostly in *E. coli* and *K. pneumoniae* and to a lesser extent in other enterobacterial species (*16*,*17*). The level of resistance to carbapenems of NDM-1 producers may vary (Table 1). Plasmids carrying the $bla_{\text{NDM-1}}$ gene are diverse and can harbor a high number of resistance genes associated with other carbapenemase genes (oxacillinase-48 [OXA-48] types, VIM types), plasmid-mediated cephalosporinase genes, ESBL genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase), rifampin (rifampin-modifying enzymes) and sulfamethoxazole resistance genes as a source of multidrug resistance and pandrug resistance (*16*,*17*) (Figure 2, panel B). The association of such a high number of resistance genes in single isolates has been rarely observed, even among the other carbapenemase producers. Many NDM-1 producers remain susceptible only to tigecycline, colistin (Figure 2, panel B), and to a lesser extent fosfomycin (*16*,*17*).

Compared with other carbapenemases, NDM-1 has several characteristics that are deeply disconcerting for public health worldwide. These characteristics are 1) occurrence of the $bla_{\text{NDM-1}}$ gene not in a single species but in many unrelated species and its spread in the environment, at least in the Indian subcontinent (*18*); 2) frequent acquisition by *K. pneumoniae,* a typical nosocomial pathogen, but also by *E. coli* that is by far the main (community-acquired) human pathogen; and 3) size of the reservoir—the Indian subcontinent has >1.4 billion persons. In certain areas in Pakistan, ≤20% of the population may carry NDM-1 producers (P. Nordmann, unpub. data).

Of particular concern, NDM-1 has been identified in *E. coli* ST-type 131 as a source of community-acquired infection (*19*), an ST type that is known to mobilize efficiently the ESBL CTX-M-15 worldwide (*20*). *E. coli* is the most common cause of diarrhea in children in India. Therefore, this organism may increase the risk of drug-resistant strains being released into the environment and further spread among humans. Accordingly, NDM-1 producers have been recently identified in tap and environmental water in New Delhi, among many unrelated gram-negative species (*18*).

Class D Enzymes of the OXA-48 Type

The first identified OXA-48 producer was from a *K. pneumoniae* strain isolated in Turkey in 2003 (*21*). Since then, OXA-48 producers have been extensively reported from Turkey as a source of nosocomial outbreaks (*22*–*26*). Their worldwide distribution now includes countries in Europe, in the southern and eastern part of the Mediterranean Sea, and Africa (*21*–*26*) (Figure 5). OXA-48

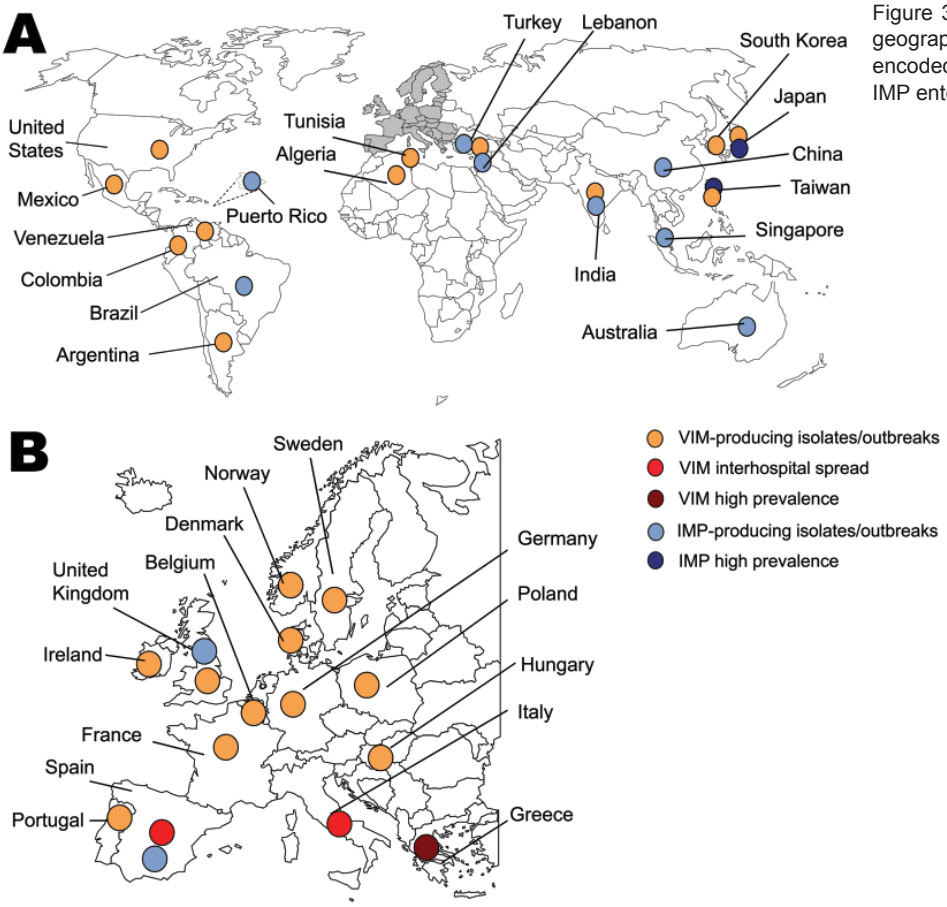

Figure 3. Worldwide (A) and European (B) geographic distribution of Verona integron–encoded metallo-β-lactamase (VIM) and IMP enterobacterial producers.

producers have not been reported from the United States and Canada. A point mutant analog of OXA-48, OXA-181, with similar carbapenemase activity, has been identified in strains from India or of Indian origin (*27*,*28*). There is an increasing trend of identification of OXA-48 producers in countries such as France, Germany, Spain, the Netherlands, and the United Kingdom through transfer of hospitalized patients from disease-endemic areas that are the source of hospital outbreaks (Figure 5).

Several OXA-48–producing clones have been identified, and dissemination of this resistance trait is associated with a 62.5-kb plasmid (previously identified as a plasmid of ≈70 kb) (*22*). OXA-48/OXA-181 are peculiar because they weakly hydrolyze carbapenems and broad-spectrum cephalosporins, such as ceftazidime, and aztreonam (*21*,*27*), (Figure 2, panel C). Their activity is not inhibited by EDTA or clavulanic acid (resistance to amoxicillin/clavulanic acid; Figure 2, panel C). Although reported in various enterobacterial species, OXA-48 producers are mostly identified in *K. pneumoniae* and *E. coli*, and the level of resistance to carbapenems is usually higher when ESBL and permeability defects are associated (*22*–*28*), (Table 1). The OXA-48–type producers are likely the most difficult carbapenemase producers to be identified. Thus, their true prevalence could be underestimated. The attributed mortality rate from infections with OXA-48 producers is unknown.

Identification of Carbapenemase Producers

The detection of carbapenemase producers in clinical infections is based first on susceptibility testing results obtained by disk diffusion or by automated systems (*29*). The Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA) breakpoints of carbapenems have been lowered substantially in 2010 for a better detection of carbapenem-resistant isolates and carbapenemase producers (Table 2). The CLSI breakpoints of carbapenems are now lower than those of the European guidelines (Table 2). Applying the CLSI breakpoints is all that is needed for making treatment decisions according to CLSI recommendations. Special tests for carbapenemase detection are recommended for epidemiology and infection issues.

However, low-level resistance and even susceptibility to carbapenems have been observed for producers of any type of carbapenemases (Table 1). We believe, as do others (*30*), that the search for carbapenemase producers should be made for any enterobacterial isolates with decreased susceptibility to carbapenems. Our opinion is based on

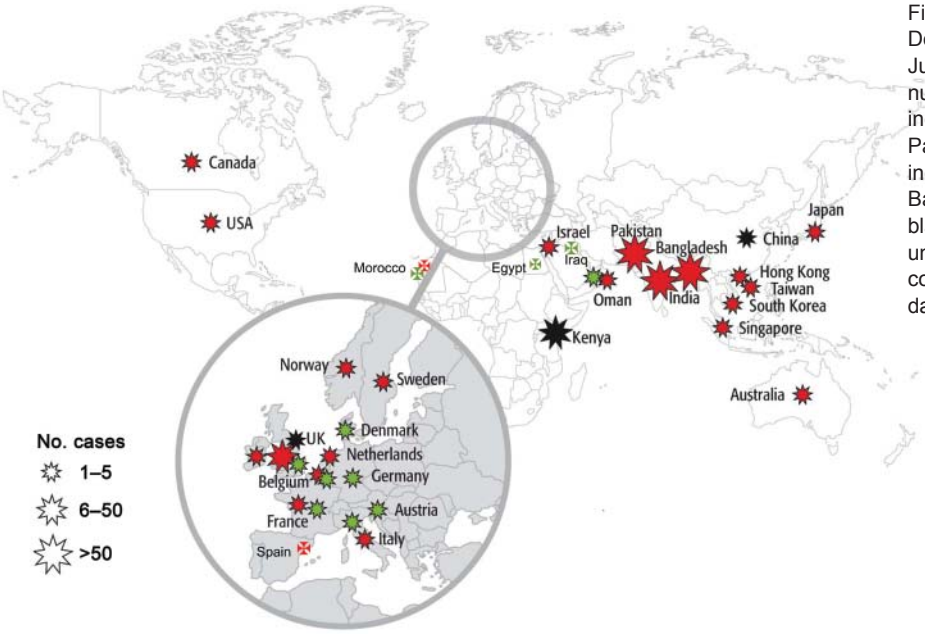

Figure 4. Geographic distribution of New Delhi metallo-β-lactamase-1 producers, July 15, 2011. Star size indicates number of cases reported. Red stars indicate infections traced back to India, Pakistan, or Bangladesh; green stars indicate infections traced back to the Balkan states or the Middle East; and black stars indicate contaminations of unknown origin. (Most of the information corresponds to published data; other data are from P. Nordmann.)

the paucity of clinical experience for treating infections caused by carbapenemase producers, on the unknown level of carbapenemase production in the site of the infection in vivo, and on the possibility of selecting in vivo for strains with increased levels of resistance to carbapenems and additional mechanisms of carbapenem resistance (carbapenemase, outer-membrane permeability defects).

Specific tests may help identify phenotypically a carbapenemase activity. The modified Hodge test based on in vivo production of carbapenemase has been suggested for detecting carbapenemase producers (*29*,*31*,*32*). However, this test is time consuming and may lack specificity (high-level AmpC producers) and sensitivity (weak detection of NDM producers) (*27*,*29*). This test may be useful for detecting KPC and OXA-48 producers (P. Nordmann, unpub. data). Boronic acid–based inhibition testing is reported to be specific for KPC detection in *K. pneumoniae* when performed with imipenem or meropenem but not with ertapenem if corresponding isolates co-produce a plasmid-mediated AmpC β-lactamase (*29*,*30*). The Etest MBL strip (bioMérieux, Solna, Sweden) is one of the methods advocated for detecting MBL producers on the basis of inhibition of MBL activity by EDTA (*12*). The Etest MBL, using imipenem and imipenem/EDTA, is efficient for detection of MBL producers with high resistance (*12*), but may be deficient for detecting MBL producers with low resistance to imipenem. No inhibition test is available for detection of OXA-48/OXA-181 producers.

Spectrophotometric assay is needed for detecting carbapenemase activity. However, this assay is time consuming, requires specific training, and does not easily discriminate between different types of carbapenemases.

The standard for identification of carbapenemases is based on use of molecular techniques, mostly PCR (*29*,*33*). A list of primers of the most prevalent carbapenemase genes identified in *Enterobacteriaceae* is shown in Table 3 (*34*). Standard conditions may be used for PCR-based detection (*34*). PCR performed on colonies may give results within 4–6 hours with excellent sensibility and specificity. Similarly, other molecular techniques, such as the Check-Points DNA technology, are useful for this purpose (*35*). Sequencing of PCR products may be of interest mostly for epidemiologic purposes. The main disadvantages of molecular-based technologies for detection of carbapenemases are their cost, the requirement of trained personal, and the absence of detection of any novel carbapenemase gene. Thus, there is an urgent need for an inexpensive, rapid, sensitive, and specific test for detection of carbapenemase activity.

The prevention of spread of carbapenemase producers relies on early detection of carriers (*29*,*33*). Patients who undergo screening should include patients who were hospitalized while abroad and then transferred to another country, and patients at risk (e.g., patients in intensive care units, transplant patients, immunocompromised patients). Screened patients should be kept in strict isolation before obtaining results of the screening (at least 24–48 hours). Because the reservoir of carbapenemase producers remains the intestinal flora, fecal and rectal swab specimens are adequate for performing this screening. Those specimens may be plated directly on screening media.

There is no universal screening medium able to detect all types of carbapenemase producers with high sensitivity and high specificity, however. Agar plates containing

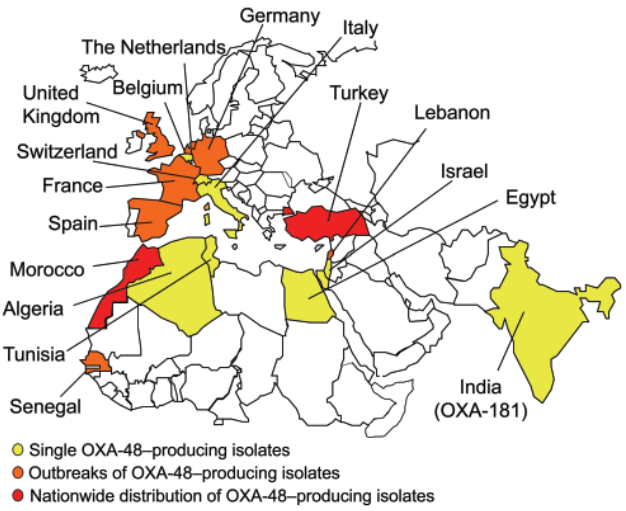

Figure 5. Geographic distribution of oxacillinase-48 (OXA-48) type producers.

imipenem at a concentration of 1 mg/L have been proposed for screening only KPC producers (*36*). We have demonstrated that a culture medium designed to screen for ESBL producers (ChromID ESBL; bioMérieux, La-Balme-Les-Grotte, France) may be used also for screening carbapenemase producers. Although this medium may lack specificity (co-detection of ESBL producers), its sensitivity is higher than a culture medium designed to screen for carbapenemase producers (CHROMagar KPC; CHROMagar, Paris, France) (*33*,*37*). The main problem remains detection of OXA-48 producers that are susceptible to cephalosporins and have low-level resistance to carbapenems when not co-producing an ESBL (Figure 2, panel C) (*37*). None of these culture media detect those OXA-48 producers (*37*).

After this screening procedure, carbapenemase producers may be identified according to the techniques described above (antibacterial drug susceptibility testing, molecular techniques). Recently, PCR-based techniques performed directly on fecal specimens have been proposed for detection of KPC and NDM-1 producers.

Table 2. Breakpoint values (MIC, mg/L) for carbapenems according to guidelines in Europe (EUCAST) and the United States (CLSI), September 2010*

	EUCAST		CLSI	
Carbapenem	S	R	S	R
Ertapenem	≤0.5	>1	≤0.25	≥1
Imipenem	≤2	>8	≤1	≥4
Meropenem	≤2	>8	≤1	≥4

*EUCAST, European Committee on Antimicrobial Susceptibility Testing (www.eucast.org/clinical_breakpoints); CLSI, Clinical and Laboratory Standards Institute; S, sensitive; R, resistant.

Conclusions

Carbapenemase producers in *Enterobacteriaceae* are not the source of specific types of clinical infections. The role of these bacteria is related to the difficult-to-treat infections rather than to expression of specific virulence traits.

We believe we are now at the edge of 2 concomitant epidemics of carbapenemase producers worldwide. The first epidemic will be caused mainly by carbapenemase producers in *E. coli* as a source of community-acquired infections. These carbapenemases are thus far primarily of the NDM and of the OXA-48 types. A few published reports of community-acquired infections caused by carbapenemase producers are available, but it is more likely that the numbers of cases in disease-endemic areas are already high. The example of the spread of ESBL producers in the community within the past 10 years shows us that a high rate of carbapenemase producers in *E. coli* may be reached rapidly worldwide. As opposed to a viral epidemic, such as pandemic (H1N1) 2009, the epidemic of carbapenemase producers cannot stop spontaneously. Such community-based outbreaks will be difficult to control. Modulation of the factors that enhance spread of carbapenemase producers in the community is difficult because these factors are multiple and are associated with lack of hygiene, overuse and over-the-counter use of antibacterial drugs, and increased worldwide travel. In addition, many carbapenemase producers carry unrelated drug-resistance determinants. Therefore, selection pressure with structurally unrelated antibacterial drugs (not only β-lactams) may contribute to their spread.

We cannot predict either the speed of diffusion of those carbapenemase producers in the community or their prevalence at a steady state (5%–50%?). The actual prevalence of carbapenemase producers is still unknown because many countries that are likely to be their main reservoirs have not established any search protocol for their detection. The prevalence may substantially differ, depending on the country, as known with the current prevalence rate of ESBL producers in *E. coli*. The prevalence is estimated to be 3%–5% in France and >80% in India (*38*).

The second epidemic will likely be caused mainly by nosocomial carbapenemase producers in *K. pneumoniae* of all types (KPC, IMP, VIM, NDM, and OXA-48). It is likely that in certain countries high rates of different types of carbapenemase producers may already exist, for example, in Greece (VIM and KPC) and in the Indian subcontinent (NDM, KPC, OXA-181). *K. pneumoniae* will play a major role because it has been repeatedly identified to be the most common enterobacterial species for spreading ESBL genes in health care facilities during the past 30 years. It may play the same role for spreading carbapenemase producers in patients with identical risk factors (patients receiving

Table 3. Oligonucleotides used for screening of main carbapenemase genes in *Enterobacteriaceae**

Primer	Sequence, 5′ → 3′	Gene	Product size, bp
IMP-F	GGAATAGAGTGGCTTAAYTC	bla_{IMP}	232
IMP-R	TCGGTTTAAYAAAACAACCACC		
VIM-F	GATGGTGTTTGGTCGCATA	bla_{VIM}	390
VIM-R	CGAATGCGCAGCACCAG		
OXA-48-F	GCGTGGTTAAGGATGAACAC	bla_{OXA-48}	438
OXA-48-R	CATCAAGTTCAACCCAACCG		
NDM-F	GGTTTGGCGATCTGGTTTTC	bla_{NDM}	621
NDM-R	CGGAATGGCTCATCACGATC		
KPC-Fm	CGTCTAGTTCTGCTGTCTTG	bla_{KPC}	798
KPC-Rm	CTTGTCATCCTTGTTAGGCG		

*A detailed technique for PCR amplification has been reported by Poirel et al. (*34*). VIM, Verona integron–encoded metallo-β-lactamase; OXA, oxacillinase; NDM, New Delhi metallo-β-lactamase-1; KPC, *Klebsiella pneumoniae* carbapenemase.

broad-spectrum antibiotherapy, patients in intensive care units, immunocompromised patients, transplant patients, surgical patients). Early identification of carbapenemase producers in clinical infections, at the carriage state, or both, is therefore mandatory to prevent development of those hospital-based outbreaks. We believe we still can efficiently prevent emergence of hospital-based outbreaks of carbapenemase producers. A similar strategy has been implemented in northern European countries for containment of hospital-acquired methicillin-resistant *Staphylococcus aureus*, which has been useful.

The dearth of novel antibacterial drugs in the pipeline means that we must conserve the efficacy of existing antibacterial drugs as much as possible. Carbapenemase producers in *Enterobacteriaceae* are different from other multidrug-resistant bacteria in that they are susceptible to few (if any) antibacterial drugs (*39*).

No vaccines are readily available for preventing infections with carbapenemase producers. This finding is particularly true for *E. coli*, which is part of the human intestinal flora. Therefore, everything must be done to prevent infections as common as pyelonephritis from becoming life threatening because of the lack of any effective treatment.

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Plasmodium knowlesi Malaria in Humans and Macaques, Thailand

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Naturally acquired human infections with *Plasmodium knowlesi* are endemic to Southeast Asia. To determine the prevalence of *P. knowlesi* malaria in malaria-endemic areas of Thailand, we analyzed genetic characteristics of *P. knowlesi* circulating among naturally infected macaques and humans. This study in 2008–2009 and retrospective analysis of malaria species in human blood samples obtained in 1996 from 1 of these areas showed that *P. knowlesi* accounted for 0.67% and 0.48% of human malaria cases, respectively, indicating that this simian parasite is not a newly emergent human pathogen in Thailand. Sequence analysis of the complete merozoite surface protein 1 gene of *P. knowlesi* from 10 human and 5 macaque blood samples showed considerable genetic diversity among isolates. The sequence from 1 patient was identical with that from a pig-tailed macaque living in the same locality, suggesting cross-transmission of *P. knowlesi* from naturally infected macaques to humans.

Plasmodium knowlesi circulates mainly among long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*M. nemestrina*) that inhabit a wide area of Southeast Asia (*1*). Microscopy-based detection of *P. knowlesi* has failed because morphologic features of young trophozoites of *P. knowlesi* resemble those of *P. falciparum* and characteristic band-shaped growing trophozoites resemble those of *P. malariae* (*2–4*). To date, the effective tool for diagnosing *P. knowlesi* infection is PCR specific for multicopy genes, such as small subunit rRNA and mitochondrial cytochrome *b* (*3–5*).

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Human infections with *P. knowlesi* vary by geographic location (highest prevalence in Malaysian Borneo), but individual cases have been increasingly identified in countries in Southeast Asia (*6*). Our large-scale molecular-based survey of malaria in Thailand during 2006–2007 showed that *P. knowlesi* was widely distributed at a low prevalence (in 0.57% of all malaria cases identified) in several malaria-endemic areas bordering Myanmar, Cambodia, and Malaysia (*7*). Correct diagnosis of malaria has a major effect on malaria control in terms of treatment outcomes, disease transmission, and interpretation of efficiency of a given control measure.

Although malaria caused by *P. knowlesi* is generally benign and responsive to chloroquine treatment, severe and fatal cases similar to complicated *P. falciparum* malaria cases have been documented (*6,8*). To date, it has been unknown whether human infections with *P. knowlesi* in Thailand were caused by a new emergence of this parasite species or whether the parasite had been circulating cryptically with other human malaria parasites. Furthermore, it would be useful to explore spatiotemporal distribution of malaria species in humans and analyze genetic characteristics of *P. knowlesi* circulating among naturally infected macaques and humans. These data could lead to a better understanding of malaria transmission and provide information for a more effective malaria control policy at a nationwide level. Therefore, we sought to determine the prevalence of this simian malaria in malaria-endemic regions of Thailand.

Materials and Methods

Prospective Study and Sample Collection

Most malaria infections in Thailand occur in forests or forest fringes along its borders with other countries, and malaria transmission exhibits a bimodal pattern that

peaks in May–July and October–November (*9,10*). During October 2008–September 2009, venous or finger prick blood samples were obtained from 3,770 febrile persons (2,577 male and 1,193 female; mean age 27.4 years, range 1–87 years) who came to malaria clinics in northwestern (Tak Province, n = 1,354), eastern (Chantaburi Province, n = 401), and southern (Yala Province, n = 1,552, and Narathiwat Province, n = 463) Thailand (Figure 1). These 3,770 persons represented 12.4% of the 30,425 malaria cases in these areas during the study period (*10*). A total of 470 blood samples from these persons were negative for malaria parasites by microscopy (153 in Tak, 179 in Yala, and 138 in Narathiwat). The study was reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

Retrospective Study

A total of 210 blood samples obtained in 1996 from microscopy-positive symptomatic malaria patients (139 male and 71 female; mean age 25.1 years, range 12–72 years) living in Tak Province were included for comparative analysis. Of these samples, 143 were obtained during May–July; the remaining 67 samples were obtained during October–December. Blood samples were preserved in EDTA and stored at –40°C until use. Results of malaria species distribution during 2006–2007 from our analysis (*7*) were included for comparison.

Microscopic Diagnosis

Thin and thick smears were prepared from each blood sample, stained with Giemsa solution, and examined by experienced microscopists for ≥200 leukocytes for a thick film and ≥200 microscopic fields with a 100× objective. Microscopists were blinded to clinical information and results of PCR detection.

PCR-based Diagnosis

DNA was extracted from 200 μL of blood by using a DNA Minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Malaria species was identified by using a nested PCR with *Plasmodium* genus–specific outer primers (M18SF0: 5′-CCATTAATCAAGAACGAAAGTTAAGG-3′ and M18SR0c: 5′-TGTTGTTGCCTTAAACTTCCTTG-3′) derived from small subunit rRNA for a primary PCR (*7*). Nested PCR was conducted in separate tubes for each pair of species-specific primers: *P. falciparum*, PF18SF: 5′-CATCTTTCGAGGTGACTTTTAG-3′ and PF18SRc: 5′-GAAGAGAGAAATAGAGTAAAAAAC-3′; *P. vivax*, PV18SF: 5′-GAATTTTCTCTTCGGAGTTTATTC-3′ and PV18SRc: 5′-GTAGAAAAGGGAAAGGGAAACTGTTA-3′; *P. malariae*, PM18SF: 5′-GAGACATTCATATATATGAGTG-3′ and PM18SRc: 5′-GGGAAAAGAACGTTTTTATTAAAAAAAAC-3′; *P. ovale*, PO18SF: 5′-AATTCCTTTCGGGGAAATTTCTTA-3′ and PO18SRc: 5′-GGGAAAAGGACACATTAATTGTAT-3′; and *P. knowlesi*, PK18SF: 5′-GAGTTTTTCTTTTCTCTCCGGAG-3′ and PK18SRc: 5′-GGGAAAGGAATCACATTTAACGT-3′. Thermal cycling profiles for primary and nested PCRs contained 35 and 25 cycles (94°C for 40 s, 60°C for 30 s, and 72°C for 1 min), respectively. PCR products were analyzed by 2% agarose gel electrophoresis.

Sequencing the Complete Merozoite Surface Protein 1 Gene of *P. knowlesi*

During December 2008–June 2009, a prospective survey of malaria in monkeys inhabiting Yala Province (n = 70) and Narathiwat Province (n = 566) showed that 5

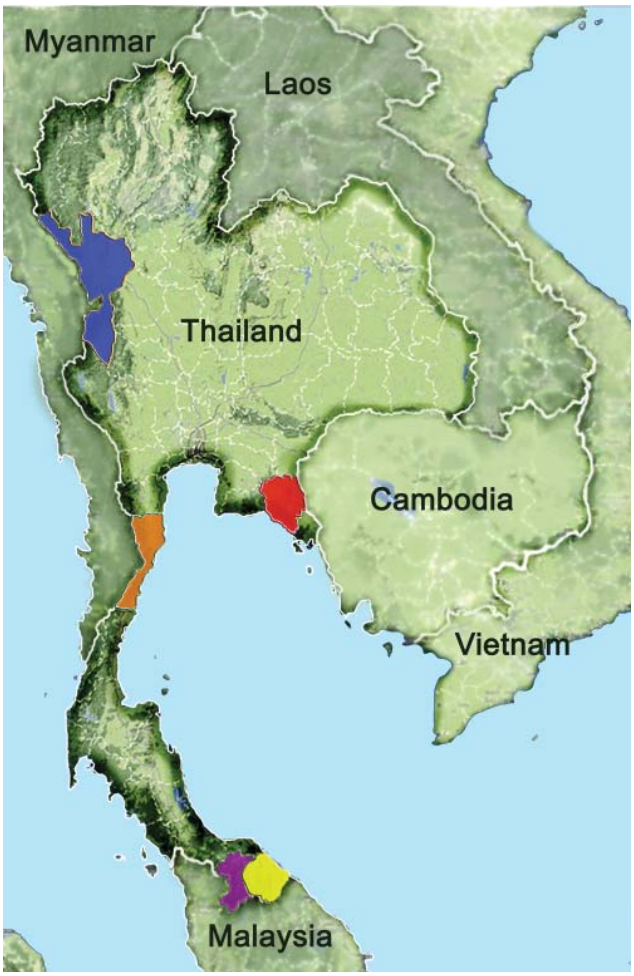

Figure 1. Provinces of Thailand where blood samples were obtained and tested for malaria, 1996–2009. Tak: blue, n = 210 in 1996, n = 681 in 2006–2007, and n = 1,216 in 2008–2009; Prachuab Khirikhan: orange, n = 215 in 2006–2007; Yala: purple, n = 286 in 2006–2007 and n = 1,408 in 2008–2009; Narathiwat: yellow, n = 370 in 2006–2007 and n = 421 in 2008–2009; and Chantaburi: red, n = 261 in 2006–2007 and n = 401 in 2008–2009.

M. nemestrina monkeys, 1 *M. fascicularis* monkey, and 1 *Semnopithecus obscurus* monkey in Narathiwat Province had *P. knowlesi* infections (*11*). The complete nucleotide sequences of the merozoite surface protein–1 gene of *P. knowlesi* (*Pkmsp-1*) from these pig-tailed macaques and humans in the current survey were obtained by direct sequencing of PCR-amplified products as described (*12*). Sequences have been deposited in GenBank under accession nos. JF837339–JF837353.

Data Analysis

Sequences were aligned by using ClustalX with minor manual adjustments made by visual inspection (*13*). Phylogenetic construction was inferred from maximum-likelihood methods by using the Hasegawa, Kishino, and Yano model with 1,000 bootstrap iterations as implemented in MEGA version 5.01 (*14*). Differences between numbers of malaria cases were computed by using the Mann-Whitney U test, χ^2 test, or Fisher exact test. A 2-tailed p value <0.05 indicated statistical significance.

Results

Malaria Species Distribution

During October 2008–September 2009, microscopic examinations of blood samples from 3,770 febrile patients showed that 3,300 had malaria parasites and 470 did not. Most malaria cases diagnosed by microscopy were either *P. falciparum* (51.55%) or *P. vivax* (48.21%); the prevalence of co-infections with both species was 0.18%. Of 432 samples containing co-infections with *P. falciparum* and *P. vivax* detected by PCR, only 3 (0.69%) samples were correctly diagnosed by microscopy. Microscopy showed negative results for 37 (8.56%) samples and failed to detect cryptic *P. falciparum* for 181 (41.90%) samples and cryptic *P. vivax* for 211 (48.84%) samples (Table 1). Conversely, 186 of 470 microscopy-negative samples were positive by nested PCR (79 *P. falciparum*, 68 *P. vivax*, 37 *P. falciparum* and *P. vivax*, 1 co-infection with *P. falciparum* and *P. malariae*, and 1 *P. knowlesi*); PCR failed to detect malaria in 46 microscopy-positive samples.

A total of 3,446 blood samples contained malaria parasite DNA and showed greater differences in species distribution and mixed species infections than did microscopy (Table 1). Mixed species infections accounted for 13.26% of all PCR-positive cases and were most common in Tak Province (Table 2). Microscopy failed to detect *P. knowlesi* in these samples. In contrast, PCR identified 23 (0.67%) patients with *P. knowlesi* infections (8 monoinfections and 15 co-infections with other *Plasmodium* species) (Table 1).

P. knowlesi in Blood Samples Obtained in 1996

PCR analysis of 210 blood samples from microscopy-positive patients in Tak Province obtained during 1996 identified 55 and 96 patients as having monoinfections with *P. falciparum* (26.19%) and *P. vivax* (45.71%), respectively. The remaining 59 patients (28.10%) had mixed species infections with *P. falciparum* and *P. vivax* (n = 50), *P. vivax* and *P. malariae* (n = 7), *P. vivax* and *P. knowlesi* (n = 1), and *P. falciparum*, *P. vivax*, and *P. malariae* (n = 1). These findings indicate that *P. knowlesi* has circulated among humans in Thailand for at least 12–13 years.

Spatial Variation

The distribution of *P. falciparum* and *P. vivax* displayed spatial variation in Thailand. PCR assays showed that *P. falciparum* and *P. vivax* contributed almost equally to malaria cases in Tak Province during 2008–2009 (a total of 50.55% and 48.68%, respectively), *P. vivax* was more

Table 1. Distribution of *Plasmodium* species in 3,770 febrile patients, Thailand, October 2008–September 2009

	Microscopy-based detection						
Species detected by PCR	*P. falciparum*	*P. vivax*	*P. malariae*	*P. ovale*	*P. falciparum* and *P. vivax*	Negative	PCR detection
P. falciparum	1,397	78	0	0	2	79	1,556
P. vivax	52	1,301	0	0	1	68	1,422
P. malariae	1	1	1	0	0	0	3
P. ovale	0	0	0	0	0	0	0
P. knowlesi	3	3	1	0	0	1	8
P. falciparum and *P. vivax*	211	181	0	0	3	37	432
P. falciparum and *P. malariae*	1	0	0	0	0	1	2
P. falciparum and *P. ovale*	3	0	0	0	0	0	3
P. falciparum and *P. knowlesi*	6	0	0	0	0	0	6
P. vivax and *P. malariae*	0	3	0	0	0	0	3
P. vivax and *P. ovale*	1	1	0	0	0	0	2
P. vivax and *P. knowlesi*	3	1	0	0	0	0	4
P. falciparum, P. vivax, and *P. knowlesi*	2	3	0	0	0	0	5
Negative	21	19	0	0	0	284	324
Total	1,701	1,591	2	0	6	470	3,770

Table 2. Distribution of *Plasmodium* species by malaria-endemic areas of Thailand, October 2008–September 2009*

Infection	Region, no. (%)				Total no. (%)
	Northwestern (Tak)	Eastern (Chantaburi)	Southern (Yala)	Southern (Narathiwat)	
Monoinfection	989 (81.33)	351 (87.53)	1,297 (92.12)	352 (83.61)	2,989 (86.74)
P. falciparum	507 (41.69)	27 (6.73)	810 (57.53)	212 (50.36)	1,556 (45.15)
P. vivax	481 (39.56)	320 (79.80)	485 (34.45)	136 (32.30)	1,422 (41.27)
P. malariae	1 (0.08)	1 (0.25)	0	1 (0.24)	3 (0.09)
P. ovale	0	0	0	0	0
P. knowlesi	0	3 (0.75)	2 (0.14)	3 (0.71)	8 (0.23)
Co-infection	227 (18.67)	50 (12.47)	111 (7.88)	69 (16.39)	457 (13.26)
P. falciparum and *P. vivax*	217 (17.85)	46 (11.47)	105 (7.46)	64 (15.20)	432 (12.54)
P. falciparum and *P. malariae*	1 (0.08)	0	0	1 (0.24)	2 (0.06)
P. falciparum and *P. ovale*	2 (0.16)	0	1 (0.07)	0	3 (0.09)
P. falciparum and *P. knowlesi*	2 (0.16)	0	3 (0.21)	1 (0.24)	6 (0.17)
P. vivax and *P. malariae*	1 (0.08)	0	2 (0.14)	0	3 (0.09)
P. vivax and *P. ovale*	1 (0.08)	0	0	1 (0.24)	2 (0.06)
P. vivax and *P. knowlesi*	2 (0.16)	1 (0.25)	0	1 (0.24)	4 (0.12)
P. falciparum, *P. vivax*, and *P. knowlesi*	1 (0.08)	3 (0.75)	0	1 (0.24)	5 (0.15)
Total	1,216	401	1,408	421	3,446

*Data from nested PCR. Percentages are of the total number in that region or infection category.

prevalent than *P. falciparum* in Chantaburi Province, and *P. falciparum* was more prevalent than *P. vivax* in Yala and Narathiwat Provinces. Despite a low overall prevalence of the remaining malaria species (<2%), *P. knowlesi* could be detected more often than *P. malariae* and *P. ovale* in these malaria-endemic areas (Table 3).

Temporal Variation

Distribution of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* in Thailand among samples obtained during 2006–2007 and 2008–2009 exhibited significant temporal variation (χ^2 99.9, 92.2, 24.6, and 20.5, respectively; all p values <0.0001). Conversely, distribution of *P. knowlesi* remained stable over these periods (χ^2 0.17, p = 0.68). If one considers that samples from 3 periods (1999, 2006–2007, and 2008–2009) were available for comparison only for Tak Province, the contribution of *P. knowlesi* to malaria cases was not different in all pairwise comparisons, which suggested stable prevalence of this simian malaria for more than a decade in this malaria-endemic area. In contrast, a significant difference was found in the distribution of *P. falciparum*, *P. vivax*, and *P. malariae* obtained during the 3 periods in each malaria-endemic area (p<0.05 for all pairwise comparisons), and *P. ovale* was not detected in samples obtained during 1996 but comprised 1.41% and 0.21% of samples obtained during 2006–2007 and 2008–2009, respectively (Table 3).

Prevalence of mixed species infections showed regional and seasonal variations characterized by a significantly higher prevalence during the dry season than in the rainy season in isolates from Chantaburi Province (χ^2 5.94, p = 0.015; odds ratio [OR] 2.59, 95% confidence interval [CI] 1.20–5.60). The opposite result was observed for isolates from Yala Province (χ^2 101.7, p<0.001; OR 7.2, 95% CI 4.65–10.90). No significant seasonal difference in prevalence of mixed species infections occurred in isolates from Tak Province (χ^2 1.09, p = 0.60; OR 1.09, 95% CI 0.81–1.46) and Narathiwat Province (χ^2 0.10, p = 0.76; OR 1.09, 95% CI 0.64–1.87).

Characteristics of *P. knowlesi*–infected Patients

Of 5,254 PCR-positive samples obtained during 1996, 2006–2007, and 2008–2009, *P. knowlesi* was detected in 34 patients (0.65%) (Tables 2, 3) (*7*). All patients infected

Table 3. Temporal variation in distribution of *Plasmodium* species by malaria-endemic region of Thailand, 2006–2009*

Species	% Distribution							
	Northwestern (Tak)		Eastern (Chantaburi)		Southern (Yala)		Southern (Narathiwat)	
	2006–2007, n = 681	2008–2009, n = 1,216	2006–2007, n = 261	2008–2009, n = 401	2006–2007, n = 286	2008–2009, n = 1,408	2006–2007, n = 370	2008–2009, n = 421
P. falciparum	44.54	50.55	8.42	16.74	57.14	60.50	23.31	56.82
P. vivax	52.63	48.68	91.03	81.50	41.86	38.97	76.17	41.34
P. malariae	1.16	0.21	0	0.22	0	0.13	0	0.41
P. ovale	1.41	0.21	0.27	0	0	0.07	0	0.20
P. knowlesi	0.26	0.35	0.27	1.54	1.00	0.33	0.52	1.22

*Data from nested PCR. Temporal variation in distribution in 1996 (n = 210) was 39.26 for *Plasmodium falciparum*, 57.41 for *P. vivax*, 2.96 for *P. malariae*, 0 for *P. ovale*, and 0.37 for *P. knowlesi*.

with *P. knowlesi*, including the first case-patient identified in 2000 (n = 35), had uncomplicated malaria symptoms (*3,7*). Age range of *P. knowlesi*–infected patients was 4–59 years (mean 30 years, mode 19 years, median 33.5 years), and most (73.50%) cases occurred in persons 16–45 years of age. *P. knowlesi* malaria was diagnosed in male patients ≈2× more often than in female patients (M:F ratio 2.18:1), which was similar to the sex distribution of total malaria cases (M:F ratio 2.16:1). Thus, no sex preference was observed for *P. knowlesi* (p = 0.97).

Almost half of the *P. knowlesi*–infected malaria patients acquired infections in southern Thailand near Malaysia. Monoinfections with *P. knowlesi* were observed in 10 patients whose parasite densities ranged from 0 to 145,000 parasites/μL. The remaining 24 patients had co-infections with *P. falciparum* (n = 16) or *P. vivax* (n = 9) or triple infections with *P. falciparum* and *P. vivax* (n = 5). Although the geometric mean parasite density for *P. knowlesi* moninfections was higher than that for mixed species infections, no significant difference was observed (Table 4).

Initial microscopic diagnosis identified *P. malariae* for patients with moninfections and *P. falciparum* or *P. vivax* for patients with mixed species infections. Approximately two thirds (23/35) of *P. knowlesi* malaria occurred in the rainy season. However, the ratio of *P. knowlesi*–infected patients to total malaria patients for the rainy and dry seasons was not significantly different (χ^2 0.25, p = 0.62; OR 1.20, 95% CI 0.60–2.38). Most (73.50%) human infections with *P. knowlesi* occurred in areas with macaques living nearby.

Msp-1 Gene Sequence of *P. knowlesi*

Fifteen complete *Pkmsp-1* gene sequences were analyzed for 10 isolates from humans and 5 isolates from pig-tailed macaques. These human isolates were obtained from 8 patients in the current survey (3 from Narathiwat Province, 3 from Chantaburi Province, and 2 from Yala Province) and from 2 patients from Prachuab Khirikhan Province obtained during 2006–2007 (*7*). The monkey isolates were obtained from 5 naturally infected pig-tailed macaques from Narathiwat Province during the same period as the current malaria survey in humans (*11*). Each isolate had single *Pkmsp-1* gene sequences because no superimposed results were observed in electropherograms. Size variation (range 5,430–5,613 bp) was observed among *Pkmsp-1* genes of these isolates.

Sequence analysis identified conserved and variable domains in *Pkmsp-1* genes, similar to those found in the *msp-1* gene of *P. vivax* (*15*). Nucleotide substitutions in conserved regions showed a dimorphic pattern. Phylogenetic analysis showed that human and macaque isolates had genetic diversity in the *Pkmsp-1* gene that could be located in 2 clusters with 100% bootstrap support, which confirmed dimorphism of this gene. One cluster contained 6 human isolates (BMC151, CT273, MC128, NR234, YL975, and YL978) and a monkey isolate from Narathiwat Province (isolate HB3), and the remaining isolates belonged to the other cluster (Figure 2). The *Pkmsp-1* gene sequence from 1 patient (NR280) in Narathiwat Province was identical with that isolated from a pig-tailed macaque (HB149) living in the same locality. Furthermore, isolates from 2 patients (YL975 and YL978) who lived in Yala Province had identical *Pkmsp-1* gene sequences. The patients were concurrently infected with *P. knowlesi* in the same malaria-endemic areas. The other identical sequences were found in isolates from 2 patients (CT157 and CT190) in Chantaburi Province who had onset of febrile illness a few days apart (Figure 2).

Discussion

Our PCR analysis identified spatiotemporal heterogeneity in the prevalence of *P. falciparum* and *P. vivax*, which is consistent with that of previous analyses by microscopy-based detection (*16,17*). Prevalence of *P. falciparum* and *P. vivax* in adjacent Yala and Narathiwat Provinces also showed a spatial difference in species distribution during 2006–2007 but no difference during 2008–2009. These results supported a spatiotemporal pattern on a microgeographic level as reported for other malaria-endemic areas in Thailand (*16*). Furthermore, the prevalence of *P. malariae* and *P. ovale* varied according to location and time.

Table 4. Parasite densities of patients with *Plasmodium knowlesi* monoinfection and co-infection with other malaria species, Thailand, October 2008–September 2009*

Category	Parasite density, parasites/μL†		
	Geometric mean	Ratio of geometric means‡	Range
P. knowlesi monoinfection, n = 10	4,165	NA	0–145,000
P. knowlesi co-infection with			
P. falciparum, n = 11	1,632	2.55	440–6,560
P. vivax, n = 9	1,686	2.47	320–13,120
P. falciparum and *P. vivax*, n = 5	487	8.55	320–1,520
All co-infections, n = 25	1,271	3.28	320–13,120

*NA, not applicable.
†Parasite densities between categories were not significantly different (p>0.05, by Mann-Whitney *U* test).
‡Ratio of parasite densities of monoinfection to co-infection.

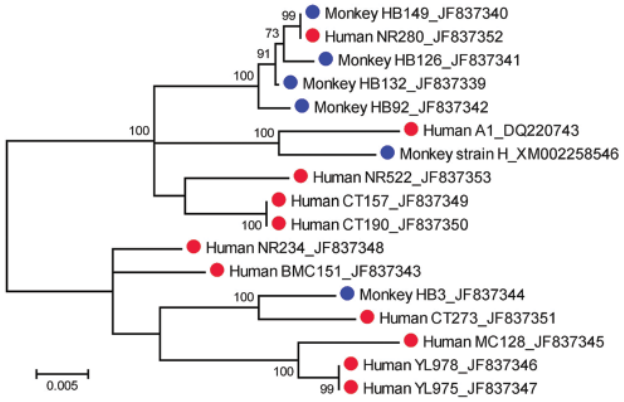

Figure 2. Maximum-likelihood tree inferred from the complete merozoite surface protein 1 gene sequences of *Plasmodium knowlesi* from humans (red circles) and macaques (blue circles). The tree is drawn to scale, and branch lengths are measured in number of substitutions per site by using MEGA version 5.01 (*14*). Bootstrap values >50% from 1,000 iterations are shown. Human isolates are from the following provinces: Narathiwat (NR280, NR234, and NR522); Yala (YL975 and YL978); Chantaburi (CT157, CT190, and CT273); and Prachuab Khirikhan (BMC151, MC128, and DQ220743). Isolates HB3, HB92, HB126, HB132, and HB149 are from macaques in Narathiwat Province. GenBank accession nos. are shown after isolate names.

Cross-border migration of infected persons, predominantly along the Thailand–Myanmar and Thailand–Cambodia borders, could contribute spatial and temporal variation in the prevalence of these malaria parasite species (*16,17*). However, migration per se could not explain similar findings in Yala and Narathiwat Provinces because almost all malaria cases in these areas were autochthonous (*10*). A dramatic increase in *P. vivax* prevalence in malaria-endemic areas bordering Cambodia during the past decade has reportedly been caused by relative changes in species distribution of local vectors and differences in vector capacity (*18–20*). Furthermore, intrinsic differences in parasite biology such as hypnozoites in *P. vivax* and *P. ovale* could contribute to additional episodes of infections if these infections are not radically treated. Therefore, spatiotemporal variation in these human cases of malaria in Thailand likely results from multiple variable factors.

Our previous survey in 2006–2007 showed that co-infections with different malaria parasite species displayed spatial variation, characterized by a high prevalence (≈23%–24%) along the Thailand–Myanmar border that contrasts with a low prevalence of 3% and 5% in malaria-endemic areas bordering Cambodia and Malaysia, respectively (*7*). Our current survey has detected different prevalences of mixed species infections than during 2006–2007 in these same malaria-endemic areas that could have been caused by different prevalences of each malaria parasite species.

Microscopy usually did not detect cryptic *P. falciparum* or cryptic *P. vivax* in samples containing both species identified by PCR (41.90% and 48.84%, respectively). When 1 of these 2 species coexisted with other malaria species (including *P. knowlesi*), only *P. falciparum* or *P. vivax* infection was diagnosed by microscopy. Failure to diagnose mixed species infections could result in repeated diagnosis and treatment, economic losses, misinterpretation of drug or vaccine efficacies, and inadequate control policies (*21*).

Co-infections with *P. falciparum* and *P. vivax* could be advantageous to hosts in terms of reduced disease severity (*22*), less chance for gametocyte carriage (*23*), and decreased parasite density (*24*). However, detrimental outcomes have been observed in other studies (*25–27*). Although parasite densities of *P. knowlesi* monoinfections and co-infections with other species were not different, the geometric mean of monoinfections was 2× that of co-infections (Table 4). Therefore, interference by a high prevalence of mixed species infections (≈14% for the current survey) and cross-species immunity may contribute to the low prevalence of *P. knowlesi* in Thailand. In contrast, mixed species infections were less prevalent in Sarawak, where most (≈91%) malaria patients had *P. knowlesi* monoinfections (*4,6*).

No difference in temporal and spatial distribution of *P. knowlesi* was found in the study areas, which suggested that transmission patterns could be different from those for human malarias caused by other parasites. The natural vectors for *P. knowlesi* in the Malay Peninsula are *Anopheles cracens* and *An. latens* mosquitoes, which are members of the Leucosphyrus group (*28,29*). The main vectors for human malaria in Thailand are *An. minimus*, *An. maculates*, and *An. dirus* mosquitoes (*30*). Although *An. dirus* mosquitoes also belong to the Leucosphyrus group and have been identified as potential vectors for *P. knowlesi* in Vietnam (*31*), this vector species has drastically decreased in abundance in all major malaria-endemic areas of Thailand during the past decade (C. Putaporntip et al., unpub. data), although feeding patterns of these mosquitoes are zoonotic rather than anthropophilic in certain areas (*32*). Therefore, transmission of *P. knowlesi* to humans could be limited and distinct from transmission by other species that cause malaria in humans in Thailand. Conversely, identification of *P. knowlesi* cryptically circulating among malaria patients in Tak Province obtained during 1996 has implied its occurrence at least 12–13 years ago in Thailand and a relatively stable prevalence. Likewise, recent analysis of archive blood samples for patients in Sarawak obtained more than a decade ago has supported the suggestion that *P. knowlesi* is not a newly emergent zoonotic malaria species in humans (*33*).

Our recent survey of simian malaria in long-tailed and pig-tailed macaques in Thailand (n = 754) showed that *P.*

knowlesi had a prevalence of 5.6% and 2.3%, respectively (*11*). Sequence analysis of *Pkmsp-1* genes from 5 *P. knowlesi*–infected macaques living near infected humans has shown that all parasites had unique sequences, which reaffirmed genetic heterogeneity of *P. knowlesi* in its natural hosts in Thailand (*7*). *P. knowlesi* isolated from a patient in Narathiwat Province shared an identical *Pkmsp-1* gene sequence with that from a pig-tailed macaque living nearby, which suggested that *P. knowlesi* could be transmitted from macaques to humans and vice versa through anopheline vectors. However, the higher prevalence of *P. knowlesi* in macaques than in humans and the chronic course of parasitemia in asymptomatic macaques could increase the likelihood of transmission from macaques to humans. Furthermore, the acute clinical course of malaria infection in humans would be rapidly eliminated by antimalarial treatment and result in a lower likelihood of additional transmission of *P. knowesi* gametocytes to vectors (*1,7*).

Identical *Pkmsp-1* gene sequences found in 2 patients in Chantaburi Province who lived in the same area and had symptomatic malaria during the same week suggests that both patients acquired the infections from a common source. Alternatively, parasites harboring this identical *Pkmsp-1* allele could predominate among infected macaques in the region. Unfortunately, no extensive survey of malaria in wild macaques in Chantaburi Province was performed during the study. Despite limited number of samples in our study, *Pkmsp-1* gene sequences from humans in eastern and southern Thailand were found in both clusters of the phylogenetic tree, which suggested that both dimorphic types are widely distributed in Thailand. Whether both dimorphic types of *Pkmsp-1* genes from infected persons co-exist in other malaria-endemic regions of Southeast Asia remains to be investigated.

A spectrum of clinical manifestations has been observed in persons infected with *P. knowlesi* (*6,34*). However, most patients with *P. knowlesi* malaria had febrile illness and associated symptoms that were indistinguishable from those caused by other malaria species (*2,3*). *P. knowlesi* requires 24 hours to complete its asexual erythrocytic cycle, which results in a unique quotidian type of fever pattern different from that for the other 4 human malaria species (*1*). However, such paroxysms have no practical value for presumptive diagnosis because characteristic fever patterns would not be observed during early phase of infections, and mixed species infections could further complicate febrile symptoms (*35*). Furthermore, *P. knowlesi* malaria is responsive to chloroquine treatment and would likely be responsive to other antimalarial drugs (*4*). Therefore, reappearance of *P. knowlesi* among patients with mixed infections after initial treatment for infection with another malaria species in Thailand seems less likely. *P. knowlesi* infections in humans caused severe symptoms and deaths in 6.5% and 1.8%, respectively, in the Kapit population in Sarawak where it comprised 70% of all malaria cases (*4*). However, if *P. knowlesi* malaria had a high prevalence of complicated symptoms, the probability of observing such severe and fatal consequences from *P. knowlesi* infections among patients in Thailand would be low.

In conclusion, human malaria caused by *P. knowlesi* has occurred in Thailand for more than a decade. Despite variations in the prevalence of all 4 human malaria species, *P. knowlesi* has shown stable prevalence rates, which suggests different transmission cycles. Human infections with *P. knowlesi* in Thailand could result from macaques, as shown by identical *Pkmsp-1* gene sequences of human and macaque origins.

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Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus Infection in England and Scotland, 2009–2010

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Oseltamivir has been widely used for pandemic (H1N1) 2009 virus infection, and by April 30, 2010, a total of 285 resistant cases were reported worldwide, including 45 in the United Kingdom. To determine risk factors for emergence of oseltamivir resistance and severe infection, a case–control study was conducted in the United Kingdom. Study participants were hospitalized in England or Scotland during January 4, 2009–April 30, 2010. Controls had confirmed oseltamivir-sensitive pandemic (H1N1) 2009 virus infections, and case-patients had confirmed oseltamivir-resistant infections. Of 28 case-patients with available information, 21 (75%) were immunocompromised; 31 of 33 case-patients (94%) received antiviral drugs before a sample was obtained. After adjusting for confounders, case-patients remained significantly more likely than controls to be immunocompromised and at higher risk for showing development of respiratory complications. Selective drug pressure likely explains the development of oseltamivir resistance, especially among immunocompromised patients. Monitoring of antiviral resistance is strongly recommended in this group.

Neuraminidase inhibitors, antiviral drugs that limit replication of influenza A and B viruses (*1*), are recommended in the United Kingdom for treatment and prophylaxis of patients at higher risk for severe or complicated influenza virus infection (*2*). During the initial containment phase of the 2009 influenza pandemic, antiviral drugs were prescribed for all patients with confirmed infections and their close contacts. During the subsequent treatment phase of the pandemic, the drugs were recommended for persons with suspected influenza virus infections who were at high risk for severe disease (*3*).

Before the 2007–08 influenza season, the development of oseltamivir-resistant influenza was rare (*4*), mainly occurring among persons who were more likely to have prolonged virus shedding, such as children (*5*) and immunocompromised patients (*6*). Patients with subtype H1N1 oseltamivir-resistant strains had the same point mutation in the viral neuraminidase gene (H275Y) that is known to confer high-level resistance to oseltamivir (*7*), but the mutation was associated with reduced infectivity and replicative ability (*8*). During the 2007–08 season, transmissible influenza A (H1N1) viruses resistant to oseltamivir (with the H275Y mutation) emerged and became predominant over susceptible subtype H1N1 viruses (*4,9*). The influenza A pandemic (H1N1) 2009 virus was initially reported as fully susceptible to the neuraminidase inhibitors (oseltamivir and zanamivir) but resistant to adamantanes, having the S31N (serine to asparagine) mutation in the M2 ion channel (*10*).

On July 8, 2009, the World Health Organization reported the first sporadic cases of oseltamivir-resistant pandemic (H1N1) 2009 infection in Denmark; Japan; and Hong Kong Special Administrative Region, People's Republic of China (*11*). By April 28, 2010, a total of 285 oseltamivir-resistant cases had been reported worldwide (*12*), including 45 in the United Kingdom. Three clusters each were reported from Wales (*13*); the United Kingdom; North Carolina, USA (*14*); and Vietnam (*15*). All of the pandemic (H1N1) 2009 oseltamivir-resistant viruses had the previously described H275Y mutation. No reassortment between the pandemic (H1N1) 2009 virus and the seasonal oseltamivir-resistant subtype H1N1 influenza strain has

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been detected (*16*–*18*), and all of the oseltamivir-resistant viruses have retained sensitivity to zanamivir.

This report describes the epidemiologic, clinical, and demographic characteristics of patients with oseltamivir-resistant pandemic (H1N1) 2009 virus infections in England and Scotland. It also identifies risk factors for severe infection and for the emergence of oseltamivir-resistant virus to inform modifications to current recommendations for the use of antiviral drugs for treatment and prophylaxis of influenza A pandemic (H1N1) 2009 virus infection.

Methods

Definition of Case-Patients and Controls

Case-patients were study participants who were hospitalized during January 4, 2009–April 30, 2010, with a confirmed case of pandemic (H1N1) 2009 virus infection with the H275Y mutation in >50% of the virus quasispecies and/or oseltamivir resistance confirmed by phenotyping of virus isolates. Controls were study participants who were hospitalized during January 4, 2009–April 30, 2010, with a confirmed case of pandemic (H1N1) 2009 virus infection with no H275Y mutation detected in the virus.

Case Detection and Collection of Epidemiologic Information

In the United Kingdom, surveillance of antiviral susceptibility of influenza viruses was performed by the Respiratory Virus Unit (RVU), Health Protection Agency. Pandemic (H1N1) 2009 infection was diagnosed from respiratory specimens by real-time reverse transcription PCR. Regional laboratories refer to RVU specimens from hospitalized case-patients with laboratory-confirmed pandemic (H1N1) 2009. The proportion referred is dependent on several factors. Emphasis is placed on the referral of positive specimens from early and late in the winter season and then a representative number during the peak influenza season. Laboratories are asked to refer equivocal specimens, specimens from patients with clinical antiviral treatment failure, and specimens from immunosuppressed patients and those who died. In addition, a proportion of community respiratory specimens from primary care clinics, selected to provide good regional coverage, were also tested for resistance. Selected specimens were tested by pyrosequencing of the neuraminidase gene to detect the presence of the H275Y mutation (*19*). The results were confirmed whenever possible by culture and phenotyping of virus isolates. Phenotypic antiviral susceptibility was determined by neuraminidase enzyme inhibition assay, using a fluorescent substrate as previously described (*20*). No patients with oseltamivir-resistant pandemic (H1N1) 2009 viruses were identified from Northern Ireland. A hospital cluster in Wales has been described separately (*13*). Therefore, this report only includes cases from England and Scotland.

For all reported cases of oseltamivir-resistant pandemic (H1N1) 2009 virus infection, epidemiologic data were gathered from the responsible clinician by the local Health Protection Unit or by Health Protection Scotland. The following patient information was collected by use of a standardized questionnaire: demographic details, clinical symptoms, complications, outcomes (hospitalization, admission to intensive care unit [ICU], death), underlying medical conditions (chronic respiratory, heart, neurologic, liver, renal diseases, diabetes, immunosuppression, pregnancy), and antiviral treatment.

Control Group

To identify risk factors for severe disease and for emergence of oseltamivir resistance, a reference control group was defined as hospitalized pandemic (H1N1) 2009 case-patients with virologically confirmed oseltamivir-sensitive infection. The control sampling frame was established by matching all virologically confirmed oseltamivir-sensitive pandemic (H1N1) 2009 specimens diagnosed by the RVU to pandemic (H1N1) 2009 cases reported to a national hospital reporting system.

Through this hospital surveillance system, microbiologists recorded standardized data for all hospital inpatients in England with laboratory-confirmed pandemic (H1N1) 2009 (*21*). Reports were made by 129 of the 160 eligible hospital trusts in England. The dataset included demographic information, underlying medical conditions, antiviral treatment, complications, and information on outcome (ICU admission, death). On the basis of surname, first name, and date of birth, a probabilistic linkage was performed between the 2,817 subtype H1N1 infections recorded in the hospital database and the 3,479 oseltamivir-sensitive pandemic (H1N1) 2009 virus infections confirmed during April 27, 2009–April 30, 2010 (Figure). This method resulted in the selection of 346 study controls. Controls were pandemic (H1N1) 2009 patients infected with oseltamivir-sensitive viruses. All controls had been hospitalized in England and had available clinical information. Recommendations and clinical practice for hospitalization of pandemic (H1N1) 2009 patients were broadly similar in England and Scotland; thus, we assume that this reference group is representative of all pandemic (H1N1) 2009 patients hospitalized in England and Scotland.

Study Design and Statistical Analysis

To assess the representativeness of the case-patients whose specimens were tested for antiviral susceptibility and to identify any potential selection bias, our control group was compared with pandemic (H1N1) 2009 patients who were recorded in the hospital database as not having been

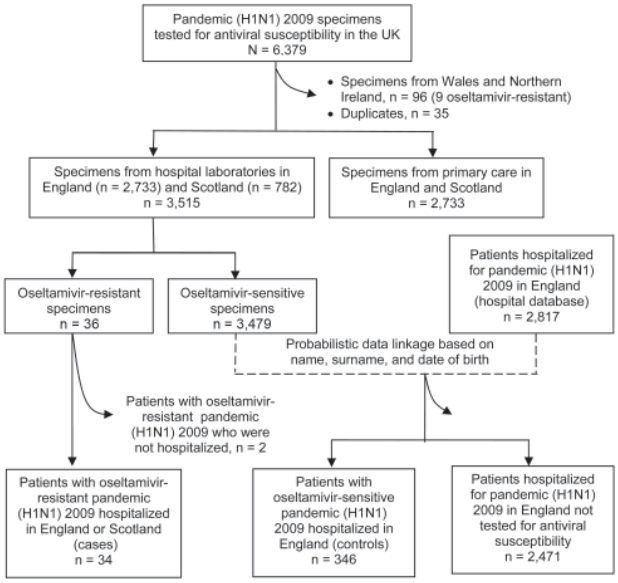

Figure. Flow chart showing testing of specimens from persons with confirmed pandemic (H1N1) 2009 infection for antiviral susceptibility, United Kingdom, April 27, 2009–April 30, 2010.

tested for antiviral susceptibility. To assess differences in distribution of possible risk factors (age, sex, underlying medical conditions) and outcomes, the χ^2 or Fisher exact test for small numbers was used.

A case–control study was conducted to compare the hospitalized pandemic (H1N1) 2009 patients with oseltamivir-resistant virus infections with hospitalized pandemic (H1N1) 2009 patients with oseltamivir-sensitive virus infections in terms of underlying medical conditions and outcomes. To estimate the association between emergence of resistance and risk factors, we calculated crude odds ratios (ORs) and 95% confidence intervals (CIs). ORs were adjusted for possible confounders by using a step-up logistic regression model. For each variable, missing data were removed from the denominator. Data analysis was performed by using Stata version 11.0 (StataCorp LP, College Station, TX, USA).

Ethical Approval

This study was conducted under National Health Service (NHS) Act 2006 (section 251), which provides statutory support for disclosure of such data by the NHS and their processing by the Health Protection Agency for the purposes of communicable disease control. Ethical approval was not required, and informed consent was not sought. Health Protection Scotland remains embedded as part of the NHS, in which the sharing of outbreak and investigation data are undertaken as part of their role in the coordination of national outbreaks.

Results

During April 27, 2009–April 30, 2010, RVU tested 6,379 pandemic (H1N1) 2009 specimens for antiviral susceptibility (*22*). Among 3,515 pandemic (H1N1) 2009 specimens sent by hospital laboratories in England and Scotland, 36 (1%) were oseltamivir resistant and 3,479 (99%) were oseltamivir sensitive (Figure). All samples from primary care clinics were oseltamivir-sensitive.

For the 36 oseltamivir-resistant samples from case-patients, the H275Y mutation was detected by pyrosequencing of the neuraminidase gene. The diagnosis was confirmed by phenotyping for 13 of these patients (36.1%) but was not confirmed by phenotypic typing for the remaining 23 patients due to unsuitable sample type (virus inactivated) or negative culture results. All 36 specimens remained sensitive to zanamivir. Oseltamivir-resistant (H275Y) quasispecies were detected in an additional 13 patients at proportions <50% (the specimen contained a mixture of virus variants, <50% of which harbored the mutation). These patients did not progress to having clinically relevant resistance, and none of the infections could be confirmed phenotypically. For those patients who had further samples available, resistant quasispecies did not persist; thus, these 13 patients are not included further in this study.

Two of the 36 patients with an oseltamivir-resistant strain were not admitted to the hospital: both were immunosuppressed boys who had mild symptoms and recovered. For both patients, the resistant strain developed after antiviral treatment, and a pretreatment specimen (fully susceptible in 1 patient and with <50% of resistant quasispecies in the other) was available.

The remaining analyses relate to the 34 case-patients hospitalized with an oseltamivir-resistant infection who were included in the case-control study. Among these 34 case-patients, 9 (26.5%) were from Scotland and 25 (73.5%) were from England. Symptom onset of case-patients ranged from June 25, 2009, to April 13, 2010, with 3 of the 34 case-patients acquiring their infection during April 27–August 30, 2009, the spring/summer wave of the pandemic.

The 34 case-patients ranged in age from 4 months to 95 years (median 52 years, mean 43.3 years) (Table 1); 23 patients (67.6%) were male, and 11 (32.4%) were female (Table 1). Details of symptoms were available for 22/34 case-patients (64.7%). The most common symptoms were cough (n = 20, 91.0%), fever (n = 17, 77.3%), and dyspnea (n = 12, 54.5%). Rhinorrhea, myalgia, headache, and fatigue were reported for 8 case-patients (36.4%) and gastrointestinal symptoms for 6 (27.3%).

Of 25 case-patients with information available regarding complications, 21 (84.0%) reported complications: in 19 (76.0%), pneumonia or bronchitis developed, 1 (4.0%) had encephalitis, and 1 (4.0%) had acute renal failure

Table 1. Distribution and reported associations of age, sex, and underlying medical conditions of study case-patients and controls hospitalized for pandemic (H1N1) 2009, England and Scotland, April 27, 2009–April, 30, 2010*

	No. (%) case-patients, n = 34		No. (%) controls, n = 346		OR (95% CI)	
Patient characteristic	n	With characteristic	n	With characteristic	Crude	Adjusted†
Sex						
M	34	23 (67.6)	346	155 (44.8)	0.4 (0.2–0.9)	0.4 (0.2–1.2)
F	34	11 (32.4)	346	191 (55.2)		
Age group, y						
0–4	34	4 (11.8)	346	64 (18.5)	1	1
5–14	34	4 (11.8)	346	83 (23.9)	0.8 (0.2–3.2)	0.5 (0.1–3.4)
15–24	34	1 (2.9)	346	59 (17.1)	0.3 (0.1–2.5)	1
25–44	34	4 (11.8)	346	74 (21.4)	0.9 (0.2–3.6)	0.5 (0.1–3.6)
45–64	34	16 (47.1)	346	56 (16.2)	4.6 (1.4–14.5)	2.4 (0.5–11.1)
≥65	34	5 (14.7)	346	10 (2.9)	8.0 (1.8–34.9)	4.1 (0.5–31.3)
Any underlying condition	30	28 (93.3)	278	164 (60.0)	9.7(2.4–85.5)	
Respiratory	28	7 (25.0)	284	94 (33.1)	0.8 (0.3–2.1)	
Cardiac	28	3 (10.7)	273	12 (4.4)	3.0 (0.5–12.1)	
Renal	28	1 (3.6)	275	11 (4.0)	1.0 (0.0–7.4)	
Liver	28	3 (10.7)	272	3 (1.1)	12.2 (1.5–95.0)	
Neurologic	28	3 (10.7)	269	15 (5.6)	2.3 (0.4–9.1)	
Immunosuppression	28	21 (75.0)	275	19 (6.9)	35.4 (12.7–102.1)	18.1 (6.6–49.9)
Diabetes	28	4 (14.3)	276	6 (2.2)	9.0 (1.7–41.0)	
Pregnancy	28	0	301	19 (6.3)		
Other chronic disease	28	6 (21.4)	258	32 (12.4)	2.5 (0.7–7.2)	

*Case-patients were those with osteltamivir-resistant pandemic (H1N1) 2009 strains; controls were those with osteltamir-sensitive pandemic (H1N1) 2009 strains. n indicates no. patients with information available for that category. OR, odds ratio; CI, confidence interval.
†OR adjusted for age, sex, and underlying conditions (e.g., immunosuppression, chronic respiratory diseases).

related to secondary group A streptococcal infection. Of the 25 case-patients with available information, 12 (48.0%) were transferred to ICU for 6–31 days (mean 16.9 days, median 15 days).

Thirty case-patients had available information regarding underlying medical conditions, of whom 28 (93.3%) had ≥1 underlying medical condition: 21 (75.0%) were immunosuppressed, 7 (25.0%) had a chronic respiratory disease, 4 (14.3%) had diabetes, 3 (10.7%) had a chronic cardiac, liver, or neurologic condition, 2 (8.0%) were morbidly obese, and 1 (4.0%) had chronic renal disease (Table 1). All but 2 of the 21 immunosuppressed patients had a hematologic cancer, and 8 of them had undergone hematopoietic cell transplantation (Table 2).

Eleven of 30 case-patients (36.7%), ranging in age from 2 to 77 years (median 61 years, mean 51 years), have died; 7 of the 11 patients had a hematologic cancer, and the other 4 had multiple chronic diseases. For 6 patients, death was attributed to pneumonia; 2 had septicemia, and 3 had multiple organ failure.

Table 2. Type of immunosuppression, presence of hematopoietic cell transplant, and outcomes for patients with oseltamivir-resistant pandemic (H1N1) 2009, England and Scotland, April 2009–April 30, 2010*

Type of immunosuppression	No. with oseltamivir-resistant strains	No. with hematopoietic cell transplant	No. admitted to ICU	No. deaths
Leukemia				
Acute lymphocytic	2	1		
Acute myeloid	3	2	1	1
Chronic lymphocytic	5	2	2	2
No precision	1			
Lymphoma				
Non-Hodgkin	2	1	1	1
Marginal zone	1			
Mantle cell	2			1
Multiple myeloma	1	1		1
Aplastic anemia	1			1
Hematologic cancer with no precision	1	1		
TRAPS	1			
HIV	1		1	
Total	21	8	5	7

*ICU, intensive care unit; TRAPS, tumor necrosis factor receptor–associated periodic syndrome.

Information on antiviral treatment was available for 33/34 (97.1%) case-patients. In specimens from 31 of the 33 (93.9%) case-patients, collected after antiviral treatment, an oseltamivir-resistant strain was detected. A pretreatment, oseltamivir-sensitive specimen was available for 22 of these case-patients. For the remaining 2 case-patients, ages 5–9 years, neither a history of antiviral pretreatment nor contact with a case of influenza-like-illness could be found. Both patients were immunocompromised and had influenza-like illness symptoms 2–4 weeks before specimens were collected. Both patients recovered fully.

Risk Factors for Antiviral Resistance

The 346 controls with oseltamivir-sensitive strains ranged in age from 0 to 103 years (median 19, mean 24); 155 patients (44.8%) were male, and 191 (55.2%) were female (Table 3). Of these controls, 58.9% had ≥1 underlying medical condition. A chronic respiratory disease was the most common underlying condition (33.1%), and 6.9% of controls were immunosuppressed (Table 3). Of the 364 control patients, 67 (19.4%) had a respiratory complication. Of 205 controls for which information was available, 59 (28.8%) were admitted to ICU; of 322 controls for which information was available, 18 (5.6%) died (Table 3).

Controls with oseltamivir-sensitive strains did not differ significantly by age and sex from the hospitalized pandemic (H1N1) 2009 patients not tested for antiviral susceptibility (Table 3). The proportion of controls with an underlying disease, as well as those who were immunosuppressed, was lower compared with patients not tested for resistance (Table 3). Other underlying diseases were distributed equally between these 2 groups. Our reference group of patients with oseltamivir-sensitive infections, although not randomly selected, thus appears to be representative of patients hospitalized with pandemic (H1N1) 2009 virus infection and, thus, reliable for assessing risk factors associated with the development of an oseltamivir-resistant virus among persons hospitalized with pandemic (H1N1)

Table 3. Distribution of age, sex, underlying medical conditions, and outcomes among persons hospitalized for oseltamivir-sensitive pandemic (H1N1) 2009 and persons hospitalized for the disease but not tested for antiviral susceptibility, England and Scotland, April 27, 2009–April 30, 2010*

	No. (%) patients with oseltamivir-sensitive strains, n = 346		No. (%) patients not tested for antiviral susceptibility, n = 2,471			
Characteristic	n	With characteristic	n	With characteristic	χ^2	p value
Sex						
M	346	155 (44.8)	2,471	1,176 (47.6)	0.95	0.33
F	346	191 (55.2)	2,471	1295 (52.4)		
Age group, y						
0–4	346	64 (18.5)	2,471	479 (19.4)		
5–14	346	83 (24.0)	2,471	463 (18.7)	2.72	0.099
15–24	346	59 (17.1)	2,471	406 (16.4)	0.19	0.663
25–44	346	74 (21.4)	2,471	611 (24.7)	0.29	0.590
45–64	346	56 (16.2)	2,471	391 (15.8)	0.13	0.718
≥65	346	10 (2.9)	2,471	121 (4.9)	1.86	0.173
Any predisposing disease	278	164 (59.0)	1,985	1,315 (66.2)	5.67	0.017
Respiratory	284	94 (33.1)	2,198	652 (29.7)	1.41	0.235
Cardiac	273	12 (4.4)	2,170	107 (4.9)	0.15	0.699
Renal	275	11 (4.0)	2,173	69 (3.2)	0.52	0.469
Liver	272	3 (1.1)	2,170	23 (1.1)	0.004	1.000†
Neurologic	269	15 (5.6)	2,181	135 (6.2)	0.16	0.692
Immunosuppression	275	19 (6.9)	1,980	237 (12.0)	6.14	0.013
Diabetes	276	6 (2.2)	2,173	104 (4.8)	3.89	0.048
Pregnancy	301	19 (6.3)	2,216	171 (7.7)	0.75	0.387
Other chronic disease	258	32 (12.4)	1,836	262 (14.3)	0.65	0.419
Complications	346	76 (22.0)	2,471	392 (15.9)	8.15	0.004
Respiratory	346	67(19.4)	2,471	374 (15.1)	4.11	0.043
Cardiac	346	2 (0.6)	2,471	0	14.29	0.015†
Renal	346	8 (2.3)	2,471	33 (1.3)	2.02	0.155
Liver	346	1 (0.3)	2,471	0	7.14	0.123†
Neurologic	346	5 (1.4)	2,471	2 (0.08)	22.78	<0.001†
Otitis	346	0	2,471	1 (0.04)	0.14	1.000†
Other	346	12 (3.5)	2,471	22 (0.9)	16.91	<0.001
ICU admission	205	59 (28.8)	1,542	258 (16.7)	17.69	<0.001
Death	322	18 (5.6)	2,253	78 (3.5)	3.55	0.059

*n indicates no. patients with information available for that category. ICU, intensive care unit.
†By Fisher exact test.

2009. However, patients with any complication and those admitted to ICU were significantly more likely to be in the group tested for antiviral susceptibility (Table 3), meaning that this study only allowed an evaluation of the course of disease among patients with the most severe pandemic (H1N1) 2009 virus infection.

Comparison between the case-patients with oseltamivir-resistant virus infections and controls with oseltamivir-sensitive infections showed, on crude analysis, that resistance was more common among middle-aged and elderly men (Table 1). Case-patients were 9× more likely than controls to have an underlying medical condition (95% CI 2.4–85.5), particularly immunosuppression (crude OR 35.4, 95% CI 12.7–102.1). Chronic liver disease and diabetes were also significantly more likely among case-patients (crude OR 12.2, 95% CI 1.5–95.0) than controls (crude OR 9.0, 95% CI 1.7–41.0).

After adjusting for age and sex, which were confounders for underlying disease in the stratified analysis, immunosuppression remained the only variable associated with development of oseltamivir resistance (adjusted OR 18.1, 95% CI 6.6–49.9). The proportions of patients with oseltamivir-resistant strains (31/33, 94.0%) and controls with oseltamivir-sensitive strains (152/170, 89.4%) who received antiviral drugs before a specimen was obtained were not significantly different (adjusted OR 1.7, 95% CI 0.4–6.6).

Risk Factors for Severe Disease

Case-patients with oseltamivir-resistant strains were at higher risk than controls with oseltamivir-sensitive strains for complications (crude OR 18.6, 95% CI 6.0–76.2), particularly for pneumonia and bronchitis (crude OR 15.8, 95% CI 5.4–55.6) (Table 4). A higher proportion of case-patients than controls were admitted to ICU (52.0% vs. 28.8%), although the difference was not significant.

The proportion of patients who died was 9.8× higher (95% CI 3.6–25.4) among case-patients with oseltamivir-resistant strains than controls (Table 4). However, after adjusting for age, sex, immunosuppression, and chronic respiratory diseases, we found a significantly higher risk for complications, particularly for respiratory complications (OR 6.6, 95% CI 1.8–23.3), remained associated with the presence of an oseltamivir-resistant strain (Table 4).

Discussion

This report summarizes the clinical and epidemiologic characteristics of one of the largest collections of oseltamivir-resistant pandemic (H1N1) 2009 cases described in the literature. Most of the 34 case-patients hospitalized in England and Scotland during April 27, 2009–April 30, 2010, with oseltamivir-resistant pandemic (H1N1) 2009 were immunocompromised middle aged or elderly men. Selective drug pressure in a particular patient subgroup seems to have been responsible for development of the resistant strain for most case-patients. Furthermore, persons with oseltamivir-resistant pandemic (H1N1) 2009 infection were more likely than those with oseltamivir-sensitive virus infections to develop complications.

This study has several limitations. First, our reference group was a convenience sample of patients hospitalized with oseltamivir-sensitive pandemic (H1N1) 2009. Underlying medical conditions and severe outcomes are more common in hospitalized patients than patients in the community; therefore, this reference group will not be representative of all pandemic (H1N1) 2009 patients, and our results cannot be generalized to community cases. Although sporadic (*14*,*23*) and clustered (*15*) cases of oseltamivir resistance have been reported in communities in several countries, the World Health Organization has not reported widespread community circulation of oseltamivir-resistant pandemic (H1N1) 2009 virus (*24*). In the United Kingdom, more than one third of subtype H1N1 specimens tested for antiviral susceptibility were from patients from the community rather than hospitalized patients. However, only 2 of 45 patients (4.4%) with oseltamivir-resistant virus were from the community, and both cases were treatment induced. The recommendations for antiviral susceptibility testing introduced a second selection bias in this study. Our reference group was found to be representative of patients hospitalized with pandemic (H1N1) 2009 in terms of age and sex. However, the proportion of patients with underlying disease and immunosuppression was lower in the tested controls than in the nontested group. This finding

Table 4. Distribution and reported associations of outcomes (complications, ICU admission, death) for study patients and controls hospitalized for pandemic (H1N1) 2009, England and Scotland, April 27, 2009–April, 30, 2010*

	Case-patients with oseltamivir-resistant strains, n = 34		Controls with oseltamivir-sensitive strains, n = 346		OR (95% CI)	
Outcome	n	No. (%) with outcome	n	No. (%) with outcome	Crude	Adjusted†
Any complications	25	21 (84.0)	346	76 (22.0)	18.6 (6.0–76.2)	9.0 (2.4–34.3)
Respiratory complications	24	19 (79.2)	346	67 (19.4)	15.8 (5.4–55.6)	6.6 (1.8–23.3)
ICU admission	23	12 (52.01)	205	59 (28.8)	2.3 (1.0–7.1)	2.3 (0.7–7.9)
Death	30	11 (36.7)	322	18 (5.6)	9.8 (3.6–25.4)	2.2 (0.5–9.5)

*n indicates no. patients with information available for that category. ICU, intensive care unit, OR, odds ratio; CI, confidence interval.
†OR adjusted for age, sex, and underlying conditions (e.g., immunosuppression, chronic respiratory diseases).

may have led to a slight overestimation of the size of the association between these risk factors and the development of oseltamivir-resistant virus.

In addition, patients who had any complication and those admitted to ICU were overrepresented in our reference group, meaning that the course of the disease was studied among the patients with the most severe concurrent conditions who were hospitalized with pandemic (H1N1) 2009. In addition, because the number of diagnosed cases of oseltamivir-resistant pandemic (H1N1) 2009 virus infection remains limited, any associations should be interpreted carefully. Last, although information on the case-patients with oseltamivir-resistant strains was actively collected, information for the controls with oseltamivir-sensitive strains was voluntarily reported by hospital microbiologists and was therefore subject to potential reporting bias.

In contrast to findings for seasonal influenza, the initial epidemiologic findings of pandemic (H1N1) 2009 in the United Kingdom were that persons <24 years of age were more likely to become infected than persons ≥65 years of age (*25*). However, in our study, >60% of the infections with oseltamivir-resistant viruses occurred in persons ≥45 years of age. The high proportion of immunocompromised persons among the resistant cases presumably explains this age difference. In a study done in the United Kingdom, the prevalence of immunocompromised patients increased with age, from 1.5% in children and young adults >7% in persons ≥70 years of age (*26*).

In this report, 93.5% of the resistant case-patients and 58.9% of the susceptible controls had ≥1 underlying medical condition. In several other countries, the presence of ≥1 risk factor was associated with an increased risk for hospitalization (*25*,*27*). As in patients with seasonal influenza, chronic respiratory disease was the most commonly reported underlying medical condition for control patients infected with a susceptible virus. However, 70% of the resistant case-patients were immunosuppressed, and immunosuppression was the only independent variable associated with the presence of an oseltamivir-resistant virus, with most of the case-patients having received oseltamivir therapy before being diagnosed with a resistant strain. These results are consistent with several other reports in which resistance seemed to develop more frequently among severely immunosuppressed patients treated with antiviral drugs (*24*,*28–30*). Prolonged virus shedding in the setting of antiviral therapy is known to lead to increased risk for the emergence of oseltamivir-resistant seasonal influenza viruses (*6*). Instances of immunosuppressed patients with prolonged virus shedding have been documented for oseltamivir-resistant seasonal and pandemic (H1N1) 2009 influenza viruses (*31*). In addition, prophylaxis and treatment were recommended for immunocompromised patients during the 2009–10 influenza pandemic.

The clinical features of case-patients infected with oseltamivir-resistant pandemic (H1N1) 2009 virus were similar to those previously described for patients hospitalized during the pandemic (*25*,*27*): fever and cough were the most common symptoms, and ≈30% of the case-patients had gastrointestinal symptoms. Of note, dyspnea was present in 55% of case-patients, which may suggest an early lower respiratory tract infection in these patients. In this study, pneumonia was the main complication reported for patients with oseltamivir-resistant strains and those with oseltamivir-sensitive strains. Pneumonia is a usual complication of seasonal influenza, particularly among immunocompromised patients (*32*). A significant proportion of patients hospitalized with pandemic (H1N1) 2009 were also reported with pneumonia (*27*,*29*,*33*). The ability of the pandemic (H1N1) 2009 virus to replicate in the lungs, as shown in animal models (*34*), may explain the high frequency of this complication in the 2009–10 pandemic. Although the risk for such complications developing was significantly higher among patients with oseltamivir-resistant strains, this result should be interpreted carefully as no information regarding either a possible bacterial co-infection or the time of sampling during the course of illness was available.

Half of the patients infected with an oseltamivir-resistant virus were admitted to the ICU, and approximately one third died. Although the risk for developing more severe outcomes appeared higher among patients with oseltamivir-resistant strains, the multivariate analysis indicated that the presence of an underlying medical condition, especially immunosuppression or chronic respiratory disease, played a more important role in the development of such severe outcomes. In other studies (*27,35–37*), underlying concurrent conditions correlated with a high risk for ICU admission and death. Immunosuppression has already been described as an important risk factor for ICU admission and death during seasonal influenza outbreaks (*38*). A more severe outcome of pandemic (H1N1) 2009 virus infection among immunocompromised persons was also reported in several studies (*27,29,30,37,39*).

In conclusion, clinicians should be aware of the emergence of oseltamivir-resistant pandemic (H1N1) 2009 virus, particularly in immunosuppressed patients. Testing for antiviral resistance is needed, especially among this group, to ensure appropriate antiviral prescribing, minimize the risk for treatment failure, and minimize the risk of person-to-person transmission of a resistant strain. Although the selective pressure of treatment seems to be the most likely mechanism to explain the development of resistant strains, person-to-person transmission has also been demonstrated. To limit the potential for secondary

transmission of resistant virus, it is recommended that clinicians check for virus clearance at the end of treatment. Changes in the recommendations of antiviral drug use for immunocompromised patients are already implemented in the United Kingdom. Either zanamivir as monotherapy or oseltamivir combined with zanamivir should be offered as primary treatment for all immunocompromised patients. Although the immune response to vaccine can be lower in some persons, particularly those who are immunosuppressed, influenza vaccination remains the major intervention to protect immunosuppressed patients who are at risk for the development of more severe disease.

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etymologia

Plasmodium knowlesi
[plaz-mo'de-əm no-le-se]

From the Greek, *plasma*, anything formed or molded, and Robert Knowles, a physician with the Indian Medical Service, credited with discovery of the organism. Knowles himself attributed the finding of *P. knowlesi* to 2 colleagues (L.E. Napier and H.G.M. Campbell) at the School of Tropical Medicine and Hygiene in Calcutta who found the protozoan while investigating kala-azar transmission. "Knowing that we should be interested in the strain from a protozoological point of view," Knowles wrote, "[Napier] handed over the original monkey to my assistant, Dr. B.M. Das Gupta." Das Gupta maintained the strain in monkeys until he and Knowles were able to carry out human infection experiments, which they reported in 1932.

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Humans Infected with Relapsing Fever Spirochete *Borrelia miyamotoi*, Russia

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Borrelia miyamotoi is distantly related to *B. burgdorferi* and transmitted by the same hard-body tick species. We report 46 cases of *B. miyamotoi* infection in humans and compare the frequency and clinical manifestations of this infection with those caused by *B. garinii* and *B. burgdorferi* infection. All 46 patients lived in Russia and had influenza-like illness with fever as high as 39.5°C; relapsing febrile illness occurred in 5 (11%) and erythema migrans in 4 (9%). In Russia, the rate of *B. miyamotoi* infection in *Ixodes persulcatus* ticks was 1%–16%, similar to rates in *I. ricinus* ticks in western Europe and *I. scapularis* ticks in the United States. *B. miyamotoi* infection may cause relapsing fever and Lyme disease–like symptoms throughout the Holarctic region of the world because of the widespread prevalence of this pathogen in its ixodid tick vectors.

Borrelia miyamotoi, discovered in Japan in 1995, belongs to the relapsing fever group of *Borrelia* (*1*). Relapsing fever borreliae infections are characterized by influenza-like illness and ≥1 relapse episode of bacteremia and fever. *B. miyamotoi* is more distantly related to *B. burgdorferi*, a group of spirochetes that includes *B. burgdorferi* s.l. strains (*B. afzelii*; *B. garinii*; and *B. burgdorferi* s.s., the causative agent of Lyme disease) (*2*,*3*). In Eurasia and North America, *B. miyamotoi* is found in a small percentage of all species of ixodid tick vectors of *B. burgdorferi*, including *Ixodes persulcatus* (*1*,*3*,*4*), *I. ricinus* (*5*–*7*), *I. scapularis* (*2*,*3*,*8*,*9*), and *I. pacificus* (*10*). It is transmitted transovarially and transstadially by ticks and coexists with *B. burgdorferi* (*2*,*3*). Recently, we discovered *B. miyamotoi* in *I. persulcatus* and *I. ricinus* ticks in the European and Asian regions of Russia. In these areas, human ixodid tick-borne infections, including those caused by *B. afzelii*, *B. garinii*, and viral tick-borne encephalitis virus (TBEV; genus *Flavivirus*) are endemic and transmitted by the same tick species.

Despite the presence of *B. miyamotoi* in vector ticks, to our knowledge, human disease caused by this spirochete has not been definitively established. We previously noted presumptive *B. miyamotoi* infection in residents of central Russia with influenza-like illness but were uncertain whether their clinical manifestations were caused by co-infecting *B. burgdorferi* s.l. species (*11*–*13*). To confirm those findings and develop initial estimates of the prevalence and severity of *B. miyamotoi* infection, we conducted a comparative cohort study. We used improved antibody assays and PCRs to compare the relative frequency and clinical manifestations of *B. miyamotoi* infection with those of *B. garinii* infection in Russia and *B. burgdorferi* infection in the United States.

Methods

Study Design

We enrolled patients admitted to Municipal Clinical Hospital No. 33 in Yekaterinburg City, Russia, from May 19 through August 25, 2009, for suspected tick-borne infection. Yekaterinburg is in the Asian part of Russia, ≈1,200 miles east of Moscow. Viral tick-borne encephalitis and acute borreliosis are highly endemic to this region. Patients with moderate or severe disease are usually hospitalized.

We compared the clinical characteristics of patients experiencing laboratory-confirmed *B. miyamotoi* infection

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with those of patients experiencing *B. garinii* infections from the same area and with those of patients who experienced *B. burgdorferi* infection in the northeastern United States. The US data came from a tick-borne diseases study conducted during 1991–2008 (*14*,*15*). For each patient at all study sites, we recorded the presence or absence of a standard set of 11 clinical manifestations. All patients signed an informed consent form in accordance with the institutional review boards of the Municipal Clinical Hospital in Yekaterinburg City or the University of Connecticut School of Medicine.

We also determined the frequency of *B. garinii*, *B. afzelii*, *B. burgdorferi*, and *B. miyamotoi* in *I. persulcatus* and *I. ricinus* ticks in Yekaterinburg and several additional regions of Russia (Figure 1). Ticks were collected by drag cloth, visually identified to species level, and analyzed by PCR to identify specific *Borrelia* species.

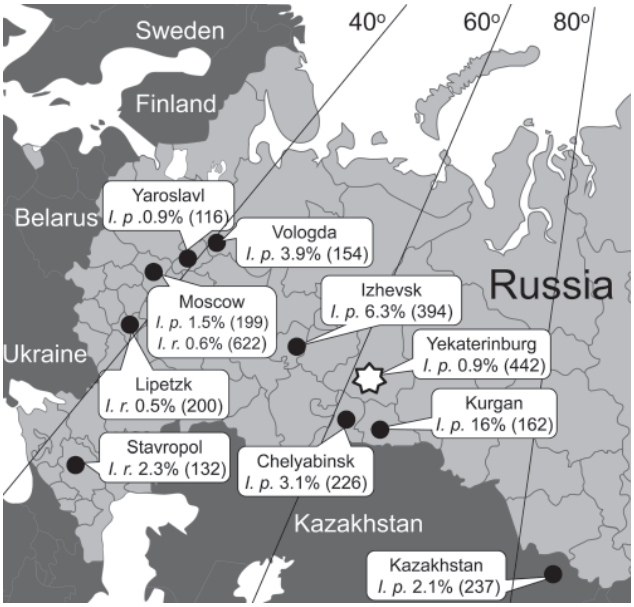

Figure 1. Percentage of *Ixodes persulcatus* (*I. p.*) and *I. ricinus* (*I. r.*) ticks infected with *Borrelia miyamotoi* in Russia. The number of ticks that were tested is given in parenthesis. Star indicates study location of human *B. miyamotoi* infection.

Case Definitions

Diagnosis of *B. miyamotoi* infection required the report of a tick bite, presence of clinical manifestations consistent with borreliosis, and laboratory evidence of *B. miyamotoi* infection. Clinical manifestations included fever, headache, chills, fatigue, vomiting, and myalgia. Confirmation of active infection consisted of amplification of *B. miyamotoi* DNA/RNA in blood by species-specific PCR and detection of anti-borreliae immunoglobulin (Ig) M in acute- and/or convalescent-phase serum samples.

In Russia, diagnosis of *B. garinii* infection required report of a tick bite, physician diagnosis of erythema migrans (EM; an expanding, ring-like erythematous rash ≥5 cm in diameter), or an influenza-like illness. Confirmation of infection consisted of amplification of *B. garinii* DNA/RNA in blood by specific PCR, followed by direct sequencing of 5S-23S ribosomal RNA (rRNA) intergenic spacers, and detection of anti-borreliae IgM in acute- and/or convalescent-phase serum samples.

In the United States, diagnosis of *B. burgdorferi* infection required a physician's diagnosis of EM or an influenza-like illness. For all cases, confirmation of infection consisted of a ≥4-fold increase in anti–*B. burgdorferi* antibody in acute- and convalescent-phase serum samples. The diagnosis of TBEV infection was based on a viral-like illness, including headache (with or without meningitis or encephalitis), amplification of TBEV RNA in blood by species-specific PCR, and/or detection of anti-TBEV IgM in an acute-phase serum sample.

Laboratory Assays

PCR

The PCR we used enabled detection of DNA and RNA sequences. DNA/RNA was extracted from 2 mL of whole venous blood with EDTA or from tick suspensions by using an AmpliSens Riboprep Kit (Central Institute of Epidemiology, Moscow, Russia) according to the manufacturer's instructions. Of the blood samples used for PCR, 81% were obtained at the time of hospital admission and 96% within 2 days of admission. To assay the inhibitory effect of blood and tick extracts on the PCR, all samples were spiked with a universal RNA recombinant control having a known number of RNA copies per milliliter. Reverse transcription of RNA to cDNA was performed by using an Amplisens Reverta-L Kit (Central Institute of Epidemiology). The cDNA samples were assayed for *B. miyamotoi* and other tick-borne pathogens by using real-time quantitative PCR (qPCR) assays in a Rotor Gene 6000 cycler (Corbett Life Science, Concorde, New South Wales, Australia).

The cDNA samples were divided into 2 aliquots, and different types of real-time qPCR were performed on each. The first used in-house primers and a probe that targeted the 16S rRNA gene of *B. miyamotoi*. The inclusion of the reverse transcription procedure improved the detection sensitivity because the 16S rRNA that also became detectable is present in higher copy numbers than the 16S rRNA gene. The detection limit of at least 5×10^3 copies/mL was determined by using positive recombinant DNA of the *B. miyamotoi* 16S rRNA gene fragment with a known number of copies. The *B. miyamotoi*–specific forward and reverse primers at 360 nmol/L were, respectively, Brm1

5′-CGCTGTAAACGATGCACACTTGGTGTTAATC-3′ and Brm2 5′-CGGCAGTCTCGTCTGAGTCCCCATCT-3′. The corresponding dye-labeled probe (final concentration 100 nmol/L) was R6G-CCTGGGGAGTATGTTCGCAAGAATGAAACTC-BQH1. The PCR conditions were 95°C for 15 min; followed by 10 cycles at 95°C for 20 s, 67°C for 50 s, and 72°C for 20 s; then by 40 cycles at 95°C for 20 s, 60°C for 50 s, and 72°C for 20 s. The fluorescence signal was recorded at the 60°C step for the last 40 cycles. Each run included negative controls and positive recombinant control DNA of the *B. miyamotoi* 16S rRNA gene fragment as a standard. PCR-based detection of *B. burgdorferi* s.l., *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis, Ehrlichia muris*, and TBEV was performed on the second cDNA aliquot by using a commercial multiplex PCR (AmpliSens TBEV, *B. burgdorferi* s.l., *A. phagocytophillum, E. chaffeensis/E. muris*-FL; Central Institute of Epidemiology) (*16*), according to the manufacturer's instructions. This assay was designed to detect, but not discriminate between, *B. afzelii*, *B. burgdorferi* s.s., and *B. garinii*. The same assays were used to detect specific DNA/RNA in ticks and humans.

The specificity of *B. miyamotoi* and *B. burgdorferi* s.l. assays was confirmed by direct sequencing of flagellin gene fragments and/or 16S rRNA gene fragments and/or 5S-23S rRNA intergenic spacer amplified from blood samples of the same patients or from the same ticks (GenBank accession nos. GU797331–GU797350, JF951378–JF951392). Of the 97 borreliae sequenced, results of DNA amplification using species-specific PCR were entirely consistent with the sequencing results. Absence of false-positive PCR results means that our PCRs were highly specific.

Amplification and further direct sequencing of the *B. miyamotoi* flagellin gene were performed by using degenerate primers FLA120F 5′-AGAATTAATMGHGCWTCTGATGATG-3′ and FLA920R 5′-TGCYACAAYHTCATCTGTCATT-3′ (*2*,*5*). The 16S rRNA gene fragment was amplified and sequenced by using 2 primers pairs: first Bf1 5′-GCTGGCAGTGCGTCTTAAGC-3′ and Brsp2 5′-CCTTACACCAGGAATTCTAACTTCCYCTAT-3′, second Brsp1 5′-GGGGTAAGAGCCTACCAAGGCTATGATAA-3′ and Br1 5′-GCTTCGGGTACTCTCAACTC-3′ (*5*). Borrelial 5S-23S rRNA intergenic spacer was amplified and sequenced by using nested PCR with outer primers pairs IGSa 5′-CGACCTTCTTCGCCTTAAAGC-3′ and IGSb 5′-AGCTCTTATTCGCTGATGGTA-3′ and inner primers pair IGSe 5′-CCTTAAAGCTCCTAGGCATTCACCA-3′ and IGSd 5′-CGCGGGAGAGTARGTTATTGCGA-3′ (*17*). Nucleotide sequences were aligned, compared, and analyzed by using MEGA4.1 (www.megasoftware.net), ClustalW (www.clustal.org), and BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

ELISA

Serum samples collected at the time of admission and 1–2 weeks later were tested for anti-borrelial IgM and IgG. Serologic evidence of exposure to borreliae was detected by ELISA EUROIMMUN EI 2132–9601 M and EI 2132–9601–2 G (EUROIMMUN AG, Lübeck, Germany). The ELISA consisted of a mixture of whole antigens from *B. afzelii*, *B. burgdorferi*, and *B. garinii* and thus could detect but not discriminate specific antibody against any of these species. Seroconversion in patients infected with the relapsing fever borrelia *B. persica* also has been detected by EUROIMMUN assay (*18*). Serum from most *B. miyamotoi*–positive patients reacted to the antigen(s) in this assay. Anti-TBE IgM was detected by the semiquantitative EUROIMMUN ELISA EI 2661–9601 M.

Statistical Analyses

Comparisons were performed by using the Mann-Whitney *U* test (independent numeric interval variables), χ^2 test (categorical variables), and corresponding exact tests, if necessary; $p<0.01$ was statistically significant. Data were analyzed by using SPSS version 11.0.1 (SPSS Inc., Chicago, IL, USA).

Results

Study Population

Among 302 patients evaluated for presumptive tick-borne infection, *B. miyamotoi* infection was found for 51 (17%) (Table 1). Of these 51 patients, anti-borrelial IgM was found at the time of admission for 6, anti-borrelial IgM seroconversion was demonstrated 2 weeks after admission for 40, no such antibody was found for 2, and laboratory evidence of TBEV co-infection was found for 3. Only the 46 patients who had amplifiable *B. miyamotoi* DNA and anti-borrelial IgM and who were not coinfected with TBEV were included in further analyses. For 18 of these patients, anti-borrelial IgG was absent at admission but detected 2 weeks after admission; subsequent IgG testing was not performed because all patients had IgM by 2 weeks. Attempts to detect *B. miyamotoi* on standard Giemsa-stained blood smears and in culture in 6 patients yielded negative results. These tests were performed 4–6 days after onset of illness but before initiation of antimicrobial drug therapy.

B. garinii infection was diagnosed for 21 (7%) patients, all of whom had amplifiable *B. garinii* DNA/RNA (GenBank accession nos. GU797347, GU797348, GU797349, GU797350) and anti-borreliae IgM in their blood. EM was found on all but 2 *B. garinii*–infected patients.

Of the remaining 230 patients, 83 had apparent *B. burgdorferi* s.l. infection (59 had EM and anti-borreliae IgM

Table 1. Classification of suspected tick-borne infections, Yekaterinburg City, Russia, May–August 2009*

Classification	Total no. patients	No. patients with erythema migrans	Amplifiable DNA/RNA, by PCR			Antibody	
			Borrelia miyamotoi	*B. burgdorferi* s.l.	TBEV	*Borrelia* IgM	TBEV IgM
B. miyamotoi infection, confirmed	46	4	46	0	0	46	0
B. miyamotoi infection, unconfirmed	2	0	2	0	0	0	0
B. miyamotoi infection, TBEV co-infection	3	0	3	0	0	2	3
B. garinii infection, confirmed	21	19	0	21	0	21	0
B. burgdorferi s.l. infection	83	83	0	0	0	59	0
Borrelia spp. infection, unconfirmed	42	0	0	0	0	42	0
TBEV infection, confirmed	21	0	0	0	5	0	21
TBEV, *B. burgdorferi* s.l. co-infection	9	9	0	0	2	ND	9
TBEV, *Borrelia* spp. co-infection	11	0	0	0	3	11	11
Other inflammatory disease	64	0	0	0	0	0	0

*TBEV, tick-borne encephalitis virus; Ig, immunoglobulin; ND, not determined.

in acute- and/or convalescent-phase serum, and 24 had EM alone); 42 had unconfirmed *Borrelia* spp. infections with anti-borreliae IgM but lacked EM and were *Borrelia* spp. negative on PCR; 41 had TBE; 37 had fever of unknown origin after tick bite; and 27 had other diagnoses, including enteroviral infection, mononucleosis, or pyelonephritis. None of the 302 patients had any PCR-based evidence of *B. afzelii*, *A. phagocytophillum,* or *E. muris* infection.

Clinical Manifestations

Patients from Russia with *B. miyamotoi* and *B. garinii* infection and patients from the United States with *B. burgdorferi* infection were similar in age and sex. Time from tick bite to onset of symptoms was longer and time from symptom onset to hospital admission was shorter for *B. miyamotoi* patients than for *B. garinii* patients (Table 2).

More systemic manifestations, including fever and headache, were reported for *B. miyamotoi* patients than for *B. garinii* and *B. burgdorferi* patients (Table 3). Maximum temperatures measured at home and in the hospital were higher for *B. miyamotoi* patients (39.0°C, interquartile range [IQR] 38.8–39.5°C) than for *B. garinii* patients (37.6°C, IQR 38.8–39.5°C; p<0.001). Duration of fever was relatively short and did not differ significantly for *B. miyamotoi* and *B. garinii* patients (3.4 ± 1.4 and 3.3 ± 2.8 days, respectively). Body temperature began to return to reference range before antimicrobial drug therapy was initiated, as has been described for relapsing fever patients, in all but 1 *B. miyamotoi* patient. Hospital stay was longer for *B. miyamotoi* patients (median 20 days, IQR 15–22 days) than for *B. garinii* patients (median 10 days, IQR 10–13 days; p<0.001).

Although mean peripheral leukocyte and platelet counts were lower for patients with *B. miyamotoi* than *B. garinii* infection, they were within the reference range. Proteinuria and transient elevation of serum alanine aminotransferase and aspartate aminotransferase concentrations were found for 3× more *B. miyamotoi* patients than *B. garinii* patients (51% and 68% vs. 15% and 20%, respectively, p<0.01), but no nephritis or hepatitis was clinically apparent. We found similar clinical and laboratory results when we omitted from analysis the 4 *B. miyamotoi* patients with EM who might have been co-infected with *B. burgdorferi* s.l.

Therapy and Clinical Outcome

Antimicrobial drug therapy for the *B. miyamotoi* patients was started ≈1 week after admission when IgM-based serologic tests results confirmed the diagnosis (median 7 days, IQR 6–10 days). Therapy consisted of ceftriaxone, 2 g intravenously every 24 hours for 2 weeks (42 patients) or doxycycline 100 mg orally every 12 hours for 2 weeks (2 patients). Two patients received no antimicrobial drug while hospitalized; 1 later received doxycycline at home, and the other was readmitted to the hospital for relapse and received ceftriaxone. Patients with *B. garinii* infection received doxycycline (71%) or ceftriaxone (29%) immediately after admission because diagnosis of borreliosis, based on presence of EM, was made at the time of admission. *B. burgdorferi* patients all received doxycycline, 100 mg orally every 12 hours, or

Table 2. Patient characteristics and infection timeline for *Borrelia* spp. infections, by species*

Borrelia species	No. patients infected	Patient characteristics		Infection timeline, median no. days (IQR)	
		Median age, y (range)	Male sex, no. (%)	Tick bite to symptom onset	Symptom onset to hospital admission
B. miyamotoi	46	54 (21–77)	24 (52)	15 (12–16)	1 (1–2)
B. garinii	21	58 (18–87)	11 (52)	10 (7–13)†	5 (2–9)†
B. burgdorferi	92	50 (14–79)	49 (53)	NA	NA

*IQR, interquartile range; NA, not available.
†p≤0.001 in comparison with patients with *B. miyamotoi* infection.

Table 3. Clinical manifestations in patients with *Borrelia* spp. infection, Yekaterinburg City, Russia, 2009, and northeastern United States, 1991–2008*

Manifestation	% Patients			p value		
	B. miyamotoi, n = 46	*B. garinii*, n = 21	*B. burgdorferi*, n = 92	*B. miyamotoi* vs. *B. garinii*	*B. miyamotoi* vs. *B. burgdorferi*	*B. garinii* vs. *B. burgdorferi*
Individual						
EM	9	91	89	<0.001	<0.001	>0.999
Multiple EM	0	14	7	0.03	0.18	0.36
Fever†	98	67	32	0.001	<0.001	0.005
Fatigue	98	86	74	0.09	<0.001	0.4
Headache	89	57	63	0.007	0.001	0.63
Chills	35	10	43	0.04	0.36	0.005
Myalgia	59	52	63	0.8	0.71	0.46
Arthralgia	28	29	62	>0.999	<0.001	0.007
Nausea	30	10	24	0.07	0.420	0.24
Vomiting	7	5	7	>0.999	>0.999	>0.999
Neck stiffness	2	0	38	>0.999	<0.001	<0.001
Overall						
No. symptoms, mean ± SD	4.5 ± 1.4	4.2 ± 2.0	5.0 ± 2.3	0.43	0.13	0.13
No. symptoms (excluding EM and multiple EM), mean ± SD	4.5 ± 1.4	3.1 ± 1.9	4.1 ± 2.3	0.007	0.46	0.09

*EM, erythema migrans.
†Maximum axillary temperature >37.2°C for patients in Russia and maximum oral temperature >37.7°C for patients in the United States.

amoxicillin, 500 mg orally every 8 hours, for 2–4 weeks. A Jarisch-Herxheimer reaction was noted for 7 (15%) of the 46 *B. miyamotoi* patients. More such reactions might have been expected if treatment had not been delayed until ≈1 week after admission. A single course of ceftriaxone or doxycycline appeared to clear *B. miyamotoi* infection.

Relapsing Infection

Of the 46 *B. miyamotoi* patients, 5 (11%, 95% confidence interval 2%–20%) experienced relapse of febrile illness; 1 patient experienced 2 relapses before hospital admission, and 4 experienced 1 relapse after hospitalization but before the start of antimicrobial drug therapy. Thus, antimicrobial drugs might have prevented relapse in those who received this therapy. The mean time between relapses was 9 days (range 2 days to 2 weeks). The maximum fever and duration of illness did not differ significantly for the first and second episodes of illness (Figure 2). No clinical or laboratory findings indicated other infections (including blood-borne, skin, neurologic, respiratory, cardiac, gastrointestinal, and urologic) or medical conditions that could account for these febrile episodes.

B. miyamotoi in Ticks

During 2004–2007, we found *B. miyamotoi*–infected *I. persulcatus* ticks in the regions where the human cases had been noted (*11*,*13*), namely, 0.9% of 442 ticks in Yekaterinburg and 6.4% of 394 ticks in Izhevsk. *B. miyamotoi*–infected *I. persulcatus* and *I. ricinus* ticks were found in regions where human cases have not yet been identified (Figure 1). These findings were confirmed by direct sequencing of PCR DNA amplification products (GenBank accession nos. GU797336, GU797337, GU797338, GU797346, JF951378–JF951392).

Genetic Characteristics of *B. miyamotoi*

The nucleotide sequences of 16S rRNA and flagellin gene fragments of all *B. miyamotoi* isolates from humans and *I. persulcatus* ticks were almost indistinguishable from the corresponding sequences of the prototype Japan HT-31 strain (*1*) (Figure 3). *B. miyamotoi* from *I. ricinus* ticks collected in the European part of Russia were more closely related to European *B. miyamotoi* strains (*5*,*6*).

Discussion

We provide confirmatory evidence of *B. miyamotoi* infection in humans. Most patients experienced clinical manifestations similar to those caused by *B. burgdorferi* s.l. and relapsing fever *Borrelia* infections, a finding consistent with the genetic characteristics of this novel spirochete. Febrile relapses occurred in only 1 of 10 *B. miyamotoi* patients, 2 days to 1 month after the initial illness; however, early treatment may have prevented subsequent relapse for other patients. Although the febrile episodes at home might have been caused by other illnesses, the onset of fever within 2 weeks after a tick bite was consistent with the incubation period of infection with borreliae. Furthermore, no clinical or laboratory findings indicated other infections or medical conditions that could account for the febrile episodes. EM was found in ≈1 of 10 *B. miyamotoi* patients, but these patients might have had unrecognized *B. burgdorferi* s.l. co-infection. A single course of ceftriaxone or doxycycline seemed to clear *B. miyamotoi* infection. Although effective therapy is available, appropriate diagnosis and therapy are

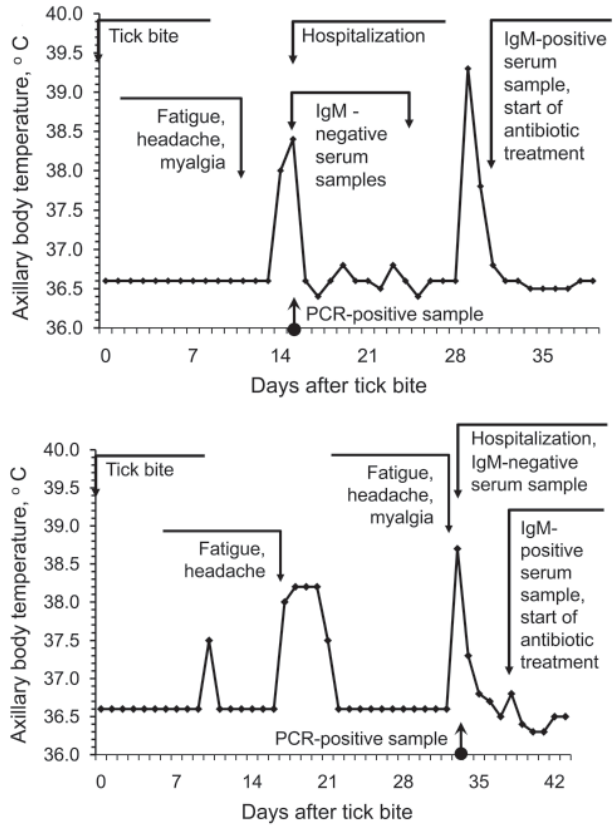

Figure 2. Examples of relapsing fever episodes in 2 patients with *Borrelia miyamotoi* infection. Arrows indicate the timing of tick bite, hospital admission, PCR testing, anti-borreliae immunoglobulin (Ig) M testing, and initiation of antimicrobial drug therapy.

complicated by lack of awareness of *B. miyamotoi* as a human pathogen, the nonspecific symptoms of infection, and the absence of standardized and widely available assays. We found no PCR-based evidence of infection caused by *B. afzelii*, *A. phagocytophilum,* or *E. muris* in the patients, although these pathogens have been detected in *Ixodes* spp. ticks in the same region (*16*). There is only anecdotal evidence of *B. afzelii* infection confirmed by culture or PCR in Russia and none in the Yekaterinburg region. Relapsing fever borreliae other than *B. miyamotoi* were not found in Yekaterinburg.

B. miyamotoi infection may cause substantial health problems in the regions of Russia where it has been found, given its relatively high incidence and associated severity of disease. On the basis of the number of patients with *B. miyamotoi* infection in Yekaterinburg Hospital in 2009 and the populations of Yekaterinburg Province (4,395,000), we estimate that the minimal incidence of *B. miyamotoi* infections is 1 case per 100,000 population. According to official federal notification during the past 10 years, ≈8,000 cases of human borreliosis occur in Russia annually (*12*). *B. miyamotoi* infection seems to constitute at least 1/4 of all clinical tick-borne borreliosis cases in Yekaterinburg. If other *Borrelia* spp.–endemic areas have a similar rate of *B. miyamotoi* infection as Yekaterinburg (and our tick data suggest that this assumption is reasonable), >1,000 *B. miyamotoi* cases might occur in Russia each year. More studies are necessary to determine if this projection is accurate.

Acute *B. miyamotoi* infection was more severe than early stage *B. burgdorferi* infection. The time from symptom onset to hospital admission was shorter, and the number of clinical manifestations was greater for patients with *B. miyamotoi* infection than with *B. garinii* infection. Relapsing febrile episodes were only reported for *B. miyamotoi* patients. Such multiple disease episodes not only have an adverse effect on a patient's health but also may result in costly medical bills, many days or weeks of lost wages, and medical misdiagnosis (*19*–*22*). Co-infection of *B. miyamotoi* with other ixodid tick–transmitted agents may increase disease severity (*15*,*23*). Additional problems that might occur with *B. miyamotoi* infection are ocular, neurologic, respiratory, cardiac, and pregnancy complications associated with relapsing fever (*19*–*22*).

Our study had several limitations. Attempts to detect *B. miyamotoi* on blood smear or in culture were not successful, although we confirmed *B. miyamotoi* infection with a combination of qPCR, genetic sequencing, clinical, and seroconversion evidence. The comparison of clinical manifestations of *Borrelia* spp. infection of patients from Russia and the United States was complicated by enrollment at different times and from different locations, although we assessed the same 11 clinical manifestations at each location. The possibility that the clinical description of our *B. miyamotoi* cases was compromised by unrecognized co-infection with *B. burgdorferi* s.l. is unlikely. The expected number of cases of co-infection depends on the prevalence of the pathogens in ticks in the region (*3*,*11*,*24*), and this number is even fewer than the 4 *B. miyamotoi* patients with EM we found. Inclusion or exclusion of these 4 cases had no effect on our comparative analysis with patients who did not have *B. miyamotoi* infection. We limited our description of *B. garinii* cases to those that were confirmed by detection of amplifiable *B. garinii* DNA/RNA, although such cases may be more severe than those in which such DNA/RNA cannot be detected (*25*,*26*). Patients with *B. burgdorferi* s.l. PCR–negative results experienced fewer symptoms and milder fever than did patients with *B. burgdorferi* s.l. PCR–positive results. Our analysis of patients with *B. miyamotoi* and *B. garinii* infection was limited to those who were hospitalized, although hospital admission policy in these regions of Russia is liberal because of concern about TBE and problems associated with *B. burgdorferi* infection.

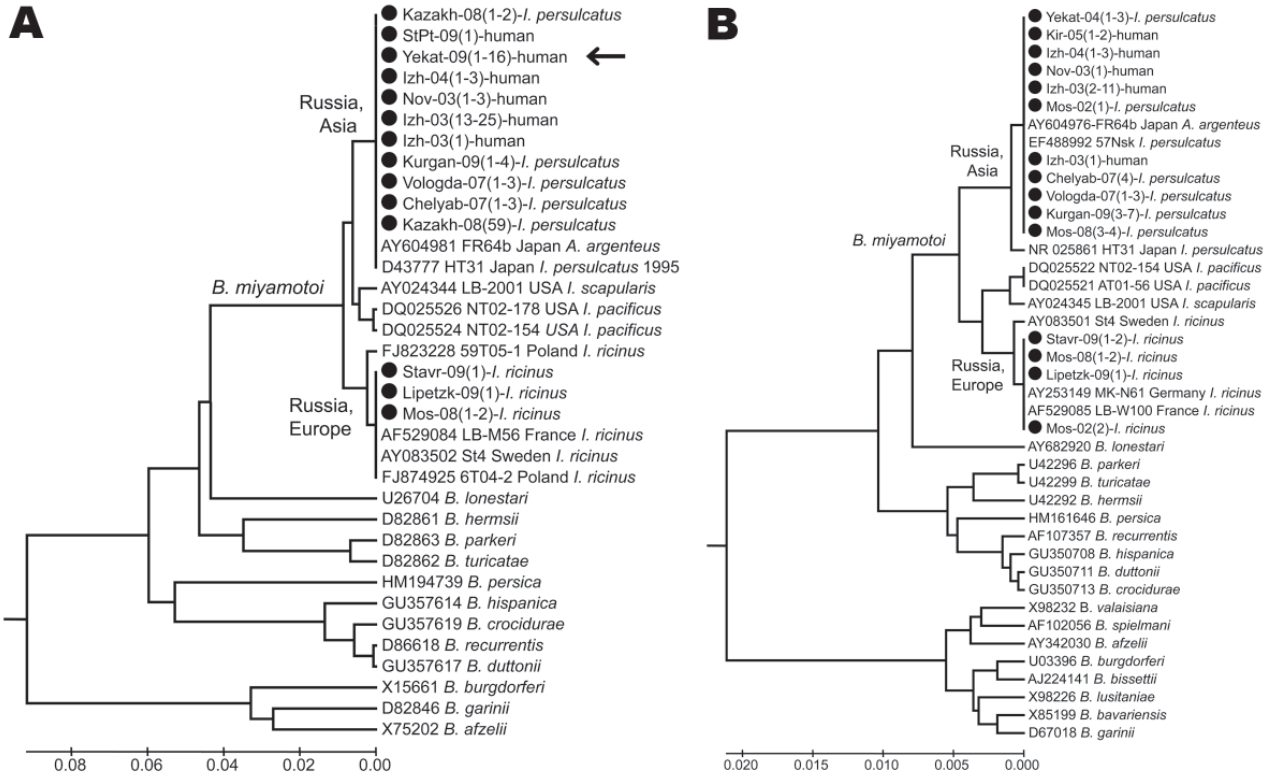

Figure 3. Phylogenetic tree of *Borrelia* spp. detected in persons and ticks, based on flagellin gene fragment (A) and16S rRNA gene fragment (B). Sequences were aligned and analyzed by using MEGA4.1 software (www.megasoftware.net). Genetic trees were constructed from the partial nucleotide sequences of the flagellin gene and the 16S rRNA gene by using the Kimura 2-parameter model and the unweighted pair group method with arithmetic mean. Arrow indicates the 16 *Borrelia* spp. from Yekaterinburg in 2009 that had the same nucleotide sequence. Circles indicate sequences that we listed in GenBank (accession nos. GU797331–GU797346 and JF951378–JF951392). Sequences for *B. burgdorferi* sensu lato and relapsing fever borreliae are shown for comparison. Scale bars indicate genetic distance.

The geographic dispersion and extent of *B. miyamotoi* disease in humans are unclear, but the infection probably occurs outside of Russia, given the comparative infection rates of vector ticks in Russia and at several locations in Europe and the United States (*2–8*). In the northeastern United States, ≈15% of all spirochetes carried by *I. scapularis* ticks are *B. miyamotoi* (*2*). Cases may remain undiagnosed because of the nonspecific nature of the illness, which might be confused with viral infections or such tick-borne infections as Lyme disease, babesiosis, anaplasmosis, or ehrlichiosis, and because of the lack of laboratory tests for confirmatory diagnosis (*19–22*).

B. miyamotoi infection may have negative health consequences, including relapsing disease that may last for months and may not respond to inappropriate antimicrobial drug therapy. The discovery of a *Borrelia* sp. that is pathogenic in humans and transmitted by an array of ixodid ticks greatly expands the potential geographic distribution of this disease (*1–11*). Further investigation of possible *B. miyamotoi* infection in humans is warranted wherever *I. pacificus, I. persulcatus, I. ricinus*, and *I. scapularis* ticks are found.

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Pandemic (H1N1) 2009 among Quarantined Close Contacts, Beijing, People's Republic of China

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We estimated the attack rate of pandemic (H1N1) 2009 and assessed risk factors for infection among close contacts quarantined in Beijing, People's Republic of China. The first 613 confirmed cases detected between May 16 and September 15, 2009, were investigated; 7,099 close contacts were located and quarantined. The attack rate of confirmed infection in close contacts was 2.4% overall, ranging from 0.9% among aircraft passengers to >5% among household members. Risk factors for infection among close contacts were younger age, being a household member of an index case-patient, exposure during the index case-patient's symptomatic phase, and longer exposure. Among close contacts with positive test results at the start of quarantine, 17.2% had subclinical infection. Having contact with a household member and younger age were the major risk factors for acquiring pandemic (H1N1) 2009 influenza virus infection. One person in 6 with confirmed pandemic (H1N1) 2009 was asymptomatic.

In early April 2009, human cases of infection with a novel influenza virus of swine origin, pandemic (H1N1) 2009 virus, were identified in the United States and Mexico, and this virus spread rapidly across the world (*1*–*3*). On June 11, 2009, the World Health Organization raised the pandemic level to 6, the highest level for pandemic alert (*4*).

Estimating attack rates is a major task in characterizing pandemic (H1N1) 2009. Some studies have reported attack rates of pandemic (H1N1) 2009 among household members and aircraft passengers (*5*–*7*). These studies suggested that the transmissibility of pandemic (H1N1) 2009 virus was low. These studies were conducted in outbreak settings, and attack rates were calculated on the basis of clinical diseases that included influenza-like illness (ILI) or acute respiratory illness (ARI) of close contacts rather than confirmed infection with pandemic (H1N1) 2009 virus. In addition, in these studies only symptomatic index and secondary cases were included. Although most infections of pandemic (H1N1) 2009 influenza virus produce ILI or ARI symptoms (*8*–*12*), subclinical infection can occur and can change the estimate of attack rate. In addition, the infectivity of asymptomatic case-patients has not been clearly defined (*13*).

Because of the high rates of illness and death among the initial case-patients with pandemic (H1N1) 2009 (*14*), the Chinese government decided to prevent and contain the rapid spread of disease through tracing and quarantine of persons who had close contact with persons with confirmed cases of pandemic (H1N1) 2009. Beijing, the capital of the People's Republic of China, took strict containment and control measures through October 2009. The Beijing municipal government implemented border entry screening, ILI screening in hospitals, health follow-up of travelers from overseas, and quarantine and testing of close contacts to identify new introduction of cases and local transmission. Public health workers conducted epidemiologic investigation of all index case-patients (including those with subclinical infections) and traced and quarantined close contacts whose residence was within the jurisdiction of Beijing. We estimated the attack rate of pandemic (H1N1) 2009 virus infection and assessed risk factors or correlates for infection among different types of close contacts, including household members and aircraft passengers.

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Methods

Confirmation of Index Cases

In 2009, under the guidance of the Beijing Center for Disease Prevention and Control (Beijing CDC), a network of 55 collaborating laboratories was established to perform reverse transcription PCR testing to confirm cases of pandemic (H1N1) 2009 (*15*). The confirmed cases included symptomatic and asymptomatic cases, and these cases were detected mainly by border entry screening, ILI screening in hospitals, health follow-up of travelers from overseas, and quarantine and testing of close contacts. Once confirmed, index case-patients were immediately quarantined in designated hospitals to receive treatment while in isolation. All the confirmed cases were required by law to be reported to Beijing and local CDCs. From May through October 2009, a detailed epidemiologic investigation was conducted for each confirmed case of pandemic (H1N1) 2009 (including symptomatic and asymptomatic cases) by Beijing and local CDCs within 6 hours after confirmation of infection. Patients with confirmed cases were interviewed about demographic characteristics, course of illness, travel and contact history, and information about close contacts. Patients with confirmed cases were categorized as having imported cases (travelers) and locally acquired cases (no travel history) on the basis of where the infection was acquired.

Definition of Close Contacts

Close contacts were defined as anyone who ever came within 2 meters of an index case-patient without the use of effective personal protective equipment (PPE) (including masks and gloves, with or without gowns or goggles) during the presumed infectious period. Trained staff from local CDCs made the determinations on the basis of field investigation. The relationships of close contacts to index case-patients were categorized as 1) spouses, 2) other household members, 3) nonrelated roommates, 4) contacts at workplace or school, 5) nonhousehold relatives, 6) passengers on the same flight, 7) friends, and 8) service persons met at public places. A close contact on an aircraft was defined as a passenger sitting within 3 rows in front and 3 rows behind the index case-patient.

All close contacts were traced and quarantined for 7 days after the most recent exposure to the index case-patient. All index case-patients detected between May 16 (the first case, the date of confirmation) and September 15, 2009 (before widespread transmission in Beijing), and their close contacts were included in this study. We excluded cluster or outbreak cases for which close contacts could not be determined clearly by epidemiologic investigation (the transmission chain was obscure).

Laboratory Screening

For each close contact, before quarantine, a pharyngeal swab specimen was collected for reverse transcription PCR testing, regardless of symptoms. A second pharyngeal swab specimen was collected for testing for pandemic (H1N1) 2009 virus if any of the following symptoms developed in a close contact during quarantine: axillary temperature ≥37.3°C, cough, sore throat, nasal congestion, or rhinorrhea.

Statistical Analysis

Data were analyzed by using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Median and range values were calculated for continuous variables, and percentages were calculated for categorical variables. Differences in attack rates were compared between subgroups of close contacts by using the χ^2 test. For the significant difference found in multiple subgroups, this test does not enable identification of which multiple subgroups are significantly different, only that across all the subgroups there are differences. The variables with $p<0.10$ in χ^2 test were included in multivariate analysis. Multivariate unconditional logistic regression analysis was conducted to determine risk factors associated with infection in close contacts. Backward logistic regression was conducted by removing variables with $p>0.10$. Odds ratios (ORs) and 95% confidence intervals were calculated for potential risk factors of infection. The Hosmer-Lemeshow goodness-of-fit test was used to assess the model fit for logistic regression. All statistical tests were 2-sided, and significance was defined as $p<0.05$.

Results

Timeliness and Intensity of Index Case Detection and Contact Tracing

A total of 613 eligible index case-patients, detected from May 16 through September 15, 2009, were included in this study. Through field epidemiologic investigations, 7,099 close contacts were traced and quarantined in Beijing. The median number of close contacts per index case per day was 7.0 persons (range 2.0–95.0 persons); the median number for an imported index case was 7.0 persons (range 1.7–95.0 persons) and for a locally acquired index case was 5.3 persons (range 1.0–25.0 persons). For the 601 symptomatic index case-patients, the median interval between illness onset and sample collection was 1.0 days (range −1.9 to 7.0 days).

Among close contacts with symptomatic infection, the median interval between illness onset and sample collection was 0.5 days. More than 85% of close contacts were quarantined within 72 hours after interview of the index case-patients. The median interval between first exposure

and quarantine was 3.4 days for the close contacts, and it was shorter, on average, for flight passenger contacts than nonpassenger contacts (1.7 days vs. 3.8 days). For symptomatic close contacts infected with pandemic (H1N1) 2009, the median of generation time (i.e., the time from illness onset in an index case to illness onset in a secondary case) were 2.4 days; it was shorter for flight passenger contacts than nonpassenger contacts (1.6 days vs. 2.5 days) (Table 1).

Characteristics of Index Case-Patients and Close Contacts

Approximately 43% of the index case-patients were women; the median age was 20 years, and 38% likely contracted pandemic (H1N1) 2009 virus locally because they had not traveled recently. Among the index case-patientss, 2% had subclinical infection. Only 18% of index case-patients had close contacts with confirmed pandemic (H1N1) 2009 (Table 2), and the total number of close contacts who were infected by the virus from 110 index case-patients was 167.

Fifty percent (3,514 of 7,032) of close contacts were women, and the median age was 27 years. Approximately 12% of close contacts were household member of index case-patients (spouse or other household member), and aircraft passengers accounted for 44% of close contacts. Approximately 61% of close contacts were exposed to symptomatic index case-patients during their symptomatic phase. About 70% were quarantined in a quarantine station (Table 2).

Attack Rate

The overall attack rate for infection among close contacts (positive test result) was 2.4% (167 of 7,099), indicating that 1 index case-patient transmitted infection to 0.27 close contacts (167 of 613) on average (reproduction number = 0.27). Among those close contacts with a positive test result, 14.4% (24 of 167) had subclinical infection; among the close contacts with positive test results at the start of quarantine, 17.2% (20 of 116) had subclinical infection.

Attack rates did not differ by index case-patient's sex ($p = 0.225$). However, attack rates differed significantly by index case-patient's age ($p = 0.022$), and the lower attack rate was found for older index case-patients. There was no significant difference in attack rates between close contacts of patients with imported cases and those with locally acquired cases ($p = 0.282$). No infection was found in close contacts exposed to index case-patients with subclinical infection, and the attack rate observed in close contacts exposed to symptomatic index case-patients during their symptomatic phase was higher ($p<0.001$). Almost identical attack rates were found among male and female close contacts ($p = 0.808$). However, attack rates were significantly different among different age groups of close contacts ($p<0.001$), and the lowest attack rate was found for those >50 years of age. The attack rates were significantly different across 8 contact types ($p<0.001$). The attack rate was 5.3% among spouses and 6.6% among other family members in the household, and was lower among other types of close contacts (Table 3).The attack rate among passengers on the same flight was low, 0.9% overall, and 1 index case-patient transmitted infection to 0.19 close contacts on a flight on average (28 of 147), and the attack rate was higher among the passengers with longer flight times (>12 hours, $p = 0.001$). The attack rate among close contacts of service persons at public places was 0.2%, and 1 index case-patient transmitted infection to 0.01 close contacts of service persons on average (1 of 113). Nonpassenger close contacts with longer exposure duration (>12 hours), compared with those with shorter duration (≥12 hours), recorded the higher attack rate ($p<0.001$) (Table 3).

Risk Factors

By multivariate analysis, age and type of contact were the major predictors of infection (Table 4). Compared with close contacts >50 years of age, those 20–50 years

Table 1. Timeliness and intensity of contact tracing for pandemic (H1N1) 2009, Beijing, People's Republic of China*

Contact type	Hours from interview of index case-patient to quarantine of close contacts, % (no./total no.)†				Median no. days (range)			
	≤24	>24–48	>48–72	>72	First exposure to quarantine†	Last exposure to quarantine†	Illness onset to sample collection‡	Generation time‡§
All close contacts	41.3 (69/167)	26.3 (44/167)	21.6 (36/167)	10.8 (18/167)	3.4 (0.2–8.1)	1.9 (0.1–6.8)	0.5 (–4.5 to 5.0)	2.4 (0.2–6.8)
Flight passenger contacts	60.7 (17/28)	28.6 (8/28)	10.7 (3/28)	0 (0/28)	1.7 (0.2–5.6)	1.2 (0.2–3.8)	0.4 (–1.0 to 1.9)	1.6 (0.3–4.8)
Nonpassenger contacts	37.4 (52/139)	25.9 (36/139)	23.7 (33/139)	12.9 (18/139)	3.8 (0.2–8.1)	1.9 (0.1–6.8)	0.6 (–4.5 to 5.0)	2.5 (0.2–6.8)

*Data available for those who were infected because more detailed information was collected in field records.
†Data for close contacts infected with pandemic (H1N1) 2009 influenza.
‡Data for symptomatic close contacts infected with pandemic (H1N1) 2009 influenza virus. Negative values indicate that the time of sample collection was before that of illness onset.
§Generation time is time from illness onset in an index case-patient to illness onset in a secondary case-patient.

Table 2. Characteristics of 613 index case-patients with pandemic (H1N1) 2009 and their close contacts, Beijing, China*

Characteristic	No. (%)
Index case-patients	
Female sex	265 (43.2)
Locally acquired cases	230 (37.5)
Subclinical infection	12 (2.0)
Infected close contacts	110 (17.9)
Close contacts, n = 7,099	
Female sex†	3,514 (50.0)
Relationship to index case-patient	
Spouse	75 (1.1)
Other household member	786 (11.1)
Nonrelated roommate	367 (5.2)
Contact at workplace or school	1,610 (22.7)
Nonhousehold relative	177 (2.5)
Passenger on the same flight	3,129 (44.1)
Friend	523 (7.4)
Service person met at public place	432 (6.1)
Contact phase	
Exposure to symptomatic index case-patient during symptomatic phase	4,305 (60.6)
Exposure to symptomatic index case-patient the day before illness onset	2,642 (37.2)
Exposure to person with subclinical index case	149 (2.1)
Type of quarantine	
Quarantine station	4,988 (70.3)
Home	2,111 (29.7)

*For index case-patients, median age (range) was 20 (1–75) y. For close contacts, median age (range) was 27 (0–99) y (data for 5,979 close contacts available).
†Data for 7,032 close contacts available.

(OR 3.42; p = 0.002) and 0–19 years of age (OR 7.76; p< 0.001) were at higher risk for infection. Other significant independent risk factors associated with infection included being a household member of a person with an index case (OR 3.83; p<0.001), being exposed to index case-patients during their symptomatic phase (OR 1.86; p = 0.003), and exposure duration >12 hours (OR 1.83; p = 0.002). Similar risk factors were observed among aircraft passengers.

Discussion

We estimated that pandemic (H1N1) 2009 virus was transmitted by 18% of index case-patients to their close contacts and that 2.4% (167 of 7,099) of close contacts we traced were infected. Our data indicate that pandemic (H1N1) 2009 virus has low transmissibility in nonoutbreak settings.

We found that 1 index case-patient transmitted infection to 0.27 close contacts on average, i.e., reproduction number = 0.27. This finding suggests that among those quarantined index case-patients, the number of persons with secondary cases who could be traced through rigorous field investigation was small and far less than the number needed for the sustainable transmission of infectious disease in the population (reproduction number >1). However, the fact that the pandemic eventually spread in Beijing indicates that contact and case tracing were far from complete, especially later in the summer and early fall of 2009. The strict control measures may have worked to some extent at the beginning but were outpaced by local transmission (*16*); the percentage of locally acquired infections ranged from <10% in June 2009 to >80% in September 2009 (data not shown).

In this study, the median number of close contacts per index case-patient per day was 7.0 persons. Although locating and quarantining these close contacts was done quickly, and stringent quarantine measures were used, which hindered implementation of control measures, the real number of close contacts was unknown and probably exceeded this number. Many close contacts were persons met in public places, including public transportation, theaters or cinemas, and shopping malls, and it is nearly impossible to trace all of the contacts. In addition, some persons who had worn PPE during contact with index case-patients were excluded from close contacts management (i.e., they were not quarantined), but because wearing PPE might not protect (or fully protect) against infection, some persons excluded might have become infected. In addition, many persons with mild and asymptomatic cases cannot be detected, but they may transmit the virus. Furthermore, the short generation time of pandemic (H1N1) 2009 shown in this study and in a previous study (*13*) could lead to the rapid accumulation of infection sources and close contacts. This rapid compounding could overwhelm response capacity and would have resulted in compromised effectiveness of containment measures. It should also be mentioned that we did not include persons with cluster or outbreak cases for whom close contacts could not be determined clearly by epidemiologic investigation to examine the basic feature of pandemic (H1N1) 2009 (e.g., generation time), and the reproduction number obtained from our data is an underestimate.

Attack rates of infection differed significantly by contact type. Among household members of index case-patients, the attack rate was the highest, as shown in the multivariate analysis after controlling for age and other factors. The most likely reason for this finding is that household members are more likely to have come into closer contact with index case-patients for a longer period with shorter distance and longer duration. Another possible reason is that household members may have some certain linkage with index cases in genetic susceptibility or living habits that would cause higher predisposition in household members than in other close contacts. This finding is similar to findings in other investigations of respiratory infectious disease (*17*).

Close contacts on flights accounted for the highest proportion of all the close contacts, in part because of how

Table 3. Attack rate for pandemic (H1N1) 2009, including subclinical infection, by characteristics of index case-patients and close contacts, Beijing, People's Republic of China

Characteristic	Attack rate, % (no. infected/total contacts)	χ^2 p value
Overall	2.4 (167/7,099)	NA*
Index case-patient		
Sex		
M	2.2 (91/4,192)	0.225
F	2.6 (76/2,907)	
Age, y		
0–19	2.7 (113/4,144)	0.022
20–50	1.9 (52/2,680)	
>50	0.7 (2/275)	
Infection source		
Imported case	2.5 (125/5,049)	0.282
Community-acquired case	2.0 (42/2,050)	
By type of exposure		
Exposure to symptomatic index case-patient in symptomatic phase	3.1 (135/4,305)	<0.001
Exposure to symptomatic index case-patient before illness onset	1.2 (32/2,642)	
Exposure to subclinical index case-patient	0 (0/149)	
Close contacts		
Sex		
M	2.3 (82/3,518)	0.808
F	2.4 (85/3,514)	
Age, y		
0–19	4.5 (82/1,837)	<0.001
20–50	2.4 (78/3,299)	
>50	0.8 (7/843)	
Relationship to index case-patient		
Spouse	5.3 (4/75)	<0.001
Other household member	6.6 (52/786)	
Nonrelated roommate	2.5 (9/367)	
Contact at workplace or school	3.0 (49/1,610)	
Nonhousehold relative	2.8 (5/177)	
Passenger on the same flight	0.9 (28/3,129)	
Friend	3.6 (19/523)	
Service person met at public place	0.2 (1/432)	
Flight time for passenger on the same flight, h		
≤12	0.4 (8/1,846)	0.001
>12	1.6 (20/1,283)	
Exposure duration of nonpassenger close contact, h		
≤12	1.9 (38/2,054)	<0.001
>12	5.3 (101/1,912)	

*Not available.

the index cases were detected and the broad definition we used for close contacts. However, the attack rate was much lower than that for other close contacts; 1 index case infected only 0.19 close contacts on flights on average. This finding indicated that the possibility of transmission of pandemic (H1N1) 2009 virus on flights was low, and the yield of tracing and quarantining of close contacts on flights was limited. Tracing contacts of service persons at public places was more difficult than tracing other categories of contacts, and the lowest attack rate (0.2%) was recorded in this category. Despite extensive measures, on average, only 0.01 infected close contacts per index case-patient were identified among service persons. Tracing the contacts of service persons at public places seems far less cost-effective. Criteria for close contacts on flights and those of service persons should be refined with respect to exposure duration and age of those exposed.

Exposure to index case-patients for >12 hours was a significant independent risk factor for infection in flight passenger contacts. This finding suggests that limiting the time of contact with persons with ILI on aircraft can reduce risk for transmission, and a long duration of exposure may be necessary for transmission to occur on aircraft.

Younger close contacts were at higher risk for infection than older ones. The possible reason was that younger persons had much closer contact with index case-patients than did older persons; another reason may be that younger persons were more susceptible to infection with pandemic

Table 4. Factors significantly associated with infection of pandemic (H1N1) 2009 virus in close contacts in multivariate analysis*

	All close contacts†		Flight passenger contacts‡		Nonflight passenger contacts§	
Factor	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
Age of close contacts, y						
>50	Reference		Reference		Reference	
20–50	3.42 (1.56–7.48)	0.002	3.13 (0.40–24.76)	0.280	2.89 (1.23–6.80)	0.015
0–19	7.76 (3.52–17.09)	<0.001	13.33 (1.77–100.22)	0.012	4.97 (2.06–12.00)	<0.001
Relationship to index case-patient						
Nonhousehold member	Reference		NA		Reference	
Household member	3.83 (2.65–5.53)	<0.001	NA	NA	2.37 (1.58–3.55)	<0.001
Type of exposure to index case-patient						
During asymptomatic phase¶	Reference		Reference		Reference	
During symptomatic phase	1.86 (1.23–2.80)	0.003	NA	NA	1.79 (1.09–2.93)	0.021
Exposure duration of close contacts, h						
≤12	Reference		Reference		Reference	
>12	1.83 (1.25–2.67)	0.002	3.41 (1.49–7.78)	0.004	NA	NA

*Variables with p<0.1 in Table 2 were included in multivariate unconditional logistic regression analysis. Hosmer-Lemeshow goodness-of-fit test was used to assess the model fit for logistic regression. OR, odd ratio; CI, confidence interval; NA, not available, indicating not included in the final model.
†One dependent variable (infection with pandemic [H1N1] 2009 virus) and 5 independent variables (age of index case-patient, type of exposure to index case-patients, age of close contacts, relationships to index case-patients, and exposure duration of close contacts) were included in multivariate analysis. One independent variable (age of index case-patient) was removed in the stepwise regression equation. The goodness-of-fit test suggested that the logistic regression model fitted well (p = 0.631).
‡One dependent variable (infection with pandemic [H1N1] 2009 virus) and 4 independent variables (age of index case-patient, type of exposure to index case-patient, age of close contacts, and exposure duration of close contacts) were included in multivariate analysis. Two independent variables (age of index case-patient and type of exposure to index case-patient) were removed in the stepwise regression equation. The goodness-of-fit test suggested that the logistic regression model fitted well (p = 0.982).
§One dependent variable (infection with pandemic [H1N1] 2009 virus) and 5 independent variables (age of index case-patient, type of exposure to index case-patient, age of close contacts, relationships to index case-patient, and exposure duration of close contacts) were included in multivariate analysis. Two independent variables (age of index case-patient and exposure duration of close contacts) were removed in the stepwise regression equation. The goodness-of-fit test suggested the logistic regression model fitted well (p = 0.751).
¶Exposed to symptomatic index case-patients before their illness onset or exposed to index case-patients who had subclinical infections.

(H1N1) 2009 virus (*18*). This finding was consistent with findings reported in other studies (*5*,*6*).

No secondary cases were found among close contacts exposed to index case-patients with subclinical infection. The attack rate among close contacts who were exposed to symptomatic index case-patients during their symptomatic phase was much higher than that among those exposed to these case-patients before their illness onset. Exposure to index case-patients during the symptomatic phase was a significant independent risk factor for infection among close contacts. These findings indicate that the infectivity of pandemic (H1N1) 2009 virus was higher after illness onset, and that the infectivity of symptomatic pandemic (H1N1) 2009 case-patients before illness onset was higher than that of persons with subclinical cases, although persons in each group were asymptomatic when in contact with other persons.

In general, the earliest infectious time for pandemic (H1N1) 2009 was considered as 1 day before illness onset (*19*). We found that index case-patients and infected close contacts shed pandemic (H1N1) 2009 virus <1 day before illness onset, which suggests that the infectious period of symptomatic persons with pandemic (H1N1) 2009 might be <1 day before illness onset.

Among close contacts with pandemic (H1N1) 2009, ≈14.4% were asymptomatic. It is noteworthy that specimens from some close contacts tested negative for pandemic (H1N1) 2009 virus before quarantine, but those persons could shed the virus during quarantine without symptoms. Such infection could not be detected, and the proportion of subclinical infection was underestimated. Therefore, we calculated the proportion of subclinical infection by cross-sectional analysis of the subclinical infection of close contacts before quarantine, and we found that ≈17% of case-patients with pandemic (H1N1) 2009 were asymptomatic.

This study has several limitations. We could not find all close contacts of persons with pandemic (H1N1) 2009 and did not know their infection status, so the infection parameters of pandemic (H1N1) 2009 that we found in this study might not be precise, especially for reproduction number, which may be underestimated to some extent. Furthermore, we could not exclude the possibility that the infected close contacts had been infected from another unknown source before quarantine started, which might influence our conclusion to some extent.

In summary, the attack rate among close contacts was low, even among household contacts. Household member and younger age were the major risk factors for infection with pandemic (H1N1) 2009 virus among close contacts. Approximately 17% of cases of pandemic (H1N1) 2009 were asymptomatic.

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Multidrug-Resistant Tuberculosis, People's Republic of China, 2007–2009

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We conducted a case–control study to investigate risk factors for multidrug-resistant tuberculosis (MDR TB) in the People's Republic of China. Genotyping analysis was used to estimate the percentage of cases from recent transmission among 100 MDR TB case-patients hospitalized during April 2007–July 2009. Molecular subtyping of isolates showed that 41% of MDR TB strains clustered. Beijing genotype was found in 94% of the MDR TB isolates and 79% of the pan-susceptible isolates. In multivariate analysis, MDR TB was independently associated with Beijing genotype, retreatment for TB, symptoms lasting ≥3 months before first evaluation at the hospital, lack of health insurance, and being a farmer (vs. being a student). MDR TB was associated with Beijing genotype and lower socioeconomic status. A large percentage of MDR TB cases seemed to result from recent transmission. Early detection, effective treatment, and infection control measures for MDR TB are needed to reduce transmission.

Multidrug-resistant tuberculosis (MDR TB), defined as resistance to at least isoniazid and rifampin, has emerged as a global public health problem (*1*). The People's Republic of China has the second greatest number of MDR TB cases in the world (*2*). According to the National Anti-Tuberculosis Drug Resistance Survey in 2007, an estimated 120,000 new MDR TB cases emerge annually in China, accounting for ≈24% of MDR TB worldwide (*3*). Although MDR TB represents only 8% of incident TB cases in China, controlling MDR TB is challenging because it is difficult to diagnose and treat (*4*). Thus, MDR TB is increasingly becoming a serious threat to TB control (*3*,*5*), and the recognition of extensively drug-resistant TB has furthered highlighted this threat (*6*,*7*).

The first pilot sites for the programmatic management of drug-resistant TB in China were established in October 2006. By the end of July 2010, similar management programs covered 41 prefectures/cities in 12 provinces in which ≈1,000 patients with MDR TB were treated with standardized treatment regimens recommended by the World Health Organization (*8*,*9*).

Mycobacterium tuberculosis acquires resistance to antimicrobial drugs through the selection of bacteria with mutations in resistance genes (*10*). Particular resistance genotypes, such as isoniazid-resistant strains from which the *katG* gene has been deleted, have been associated with decreased growth and persistence of *M. tuberculosis* in mice and guinea pigs (*11*). A recent molecular study suggests that drug-resistant strains of *M. tuberculosis* may be as transmissible as pan-sensitive strains (*12*). However, some isoniazid-resistant strains, such as those with a mutation at aa 315 of the *katG* gene, were as transmissible as drug-susceptible strains; these resistant, but equivalently transmissible, strains are typically associated with outbreaks (*13*–*15*).

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In the past decade, many studies have evaluated the role of the Beijing genotype of *M. tuberculosis* in the worldwide TB epidemic (*16*,*17*). Beijing genotype strains are emerging in Southeast Asia, former Soviet republics, the Baltic states, and South Africa and are associated with multidrug resistance (*17*–*22*). In Europe, during 2003–2006, about half of MDR TB and extensively drug-resistant TB cases were caused by recent transmission, and 85% of those cases were caused by Beijing strains; during the same period, only 6%–7% of drug-susceptible TB cases in Europe were caused by Beijing strains (*21*,*22*). As the name suggests, Beijing genotype strains are particularly prevalent in China. In a survey of 10 provinces in China, the average percentage of Beijing genotype strains was 73%, but the percentage varied substantially by region, with the highest (93%) in the Beijing region (*23*).

Genotyping studies help elucidate transmission of TB by specific strains (*17*–*20*). Since 1993, IS*6110* restriction fragment-length polymorphism typing has been considered the standard for studying the molecular epidemiology of TB (*24*). Although restriction fragment-length polymorphism typing has brought significant new insights into TB transmission, the method is technically demanding and time-consuming (*23*). Therefore, a new standard typing method using mycobacterial interspersed repetitive unit–variable-number of tandem repeats (MIRU-VNTR) in the genome was recently proposed for studying clustering and transmission (*25*). The analysis of regions of difference (RDs) in the genome of *M. tuberculosis* complex can be used to study the phylogeny of these bacteria; this approach can also be used as an alternative to the more complicated spoligotyping method for Beijing genotype strain identification (*26*,*27*).

We used the RD105 deletion detection method to identify Beijing genotype strains. We also used 24-locus VNTR typing to investigate MDR TB transmission in patients admitted to the largest TB hospital in Shandong Province during April 2007–July 2009. Our goal was to characterize the genotypes of different MDR TB strains and identify specific risk factors associated with MDR TB and MDR TB strain clustering. A study in TB patients in the same hospital during 2004–2007 showed a prevalence of MDR TB of 10.8% (*28*). Although the national guideline of the TB control program requests directly observed treatment, in which TB patients take all doses under supervision, another study in rural Shandong showed that most TB patients do not receive directly observed treatment (*29*), which poses a risk for drug resistance. In Shandong Province, the programmatic management of drug-resistant TB has been introduced only in 1 prefecture, starting in October 2008.

Methods

Sampling Method

We conducted a case–control study at Shandong Provincial Tuberculosis Hospital (SPTH). We studied all 100 MDR TB patients and 97 patients infected with pan-sensitive TB strains who were admitted during April 2007–July 2009. The patients with pan-sensitive TB were randomly selected from an electronic database with information on 974 hospitalized patients. According to the national reporting system, ≈10% of all TB cases are reported by specialized TB hospitals.

Data Collection

We obtained the following information from medical records for all study patients: sex, age, occupation, history of close contact with a TB patient (defined as a household member or colleague with TB), health insurance, TB treatment history, symptom duration before first evaluation at SPTH, presence of cavities on chest radiographs, current alcohol use, current smoking, sputum smear test results, and HIV status. During the study period, all enrolled patients were admitted to the hospital 1 time only, and the average admission time was ≈1 month.

Drug Susceptibility Testing

Sputum samples from all hospitalized TB patients in SPTH were collected before the start of TB therapy and then cultured on Lowenstein-Jensen culture medium. *M. tuberculosis* complex was identified by culturing on Lowenstein-Jensen medium containing *p*-nitrobenzoic acid, where growth indicates that the bacilli are not part of the *M. tuberculosis* complex. Isolates from all patients with *M. tuberculosis*–positive culture results were subjected to drug susceptibility testing. The first-line drugs isoniazid, rifampin, streptomycin, and ethambutol were tested for drug susceptibility on the basis of the previously described proportion method (*30*). External quality assurance on drug susceptibility testing by proficiency testing is conducted regularly by the national reference laboratory of China and the supranational reference laboratory of the Public Health Laboratory, Hong Kong Special Administrative Region, China. In addition, results of drug susceptibility testing of study samples were rechecked by the national reference laboratory.

DNA Extraction and Identification of Beijing Genotype TB Strain

DNA was extracted according to standard methods (*31*). The Beijing genotype TB strain was identified by using deletion-targeted multiplex PCR to detect the RD105 deletion (*26*,*27*). Primers were synthesized according to a

study by Brosch et al. (*26*). The upstream primer used was 5′-GGAGTCGTTGAGGGTGTTCAGCTCAGCTCAG TC-3′; the 2 different downstream primers used were 5′-CGCCAAGGCCGCATAGTCACGGTCG-3′ and 5′-GGTTGCCCACTGGTCGATATGGTGGACTT-3′. An amplified sequence length of 761 bp corresponded to a Beijing genotype strain, and a sequence length of 1,466 bp corresponded to a non-Beijing genotype.

The reaction system comprised 1 μL each of upstream and downstream primer (0.4 μmol/L), 5 μL of 2× Taq PCR MasterMix (Tiangen Biotech [Beijing] Co., Ltd., Beijing, China), and 2 ng of DNA template with sufficient double-distilled water added to bring the final volume to 10 μL. Thirty-five cycles were applied in the PCR in the following manner. Each cycle consisted of denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 65°C for 30 s, followed by 72°C for 30 s, and then extension at 72°C for 7 min, after which products were stored at 4°C.

Amplification products were analyzed by using 1% agarose gel, stained by 1 μg/mL ethidium bromide in 1× TrIS-borate-EDTA electrophoresis buffer. The standard strain H37Rv was used as a control, and we used a 100-bp DNA ladder (Takara Biotechnology [Dalian] Co., Ltd., Dalian, China) for distinguishing molecular sizes.

Genotyping

For genotyping, we used the 24-loci MIRU-VNTR method, as recommended by Supply et al. (*25*). Primers were designed as described (*25*) and synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China). Each PCR was performed with 2 ng of template DNA in a 20-μL final volume composed of 10 μL 2× PCR Master Mix (Tiangen Biotech [Beijing] Co., Ltd), 1 μL of each primer (final concentration 0.4 μmol/L), and 8 μL of distilled water.

Thermal cycling consisted of the first denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 50 s, annealing at 65°C for 50 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were stored at 4°C before further analysis.

PCR products were analyzed by electrophoresis on 1% agarose gels after staining with ethidium bromide (1 μg/mL). The sizes of the products were determined by using a gel imager with a 100-bp DNA ladder (Takara) as a reference. The number of repeats at each locus was calculated in relation to the size of the locus. H37Rv was used as reference.

Statistical Analyses

BioNumerics version 5.0 (Applied Maths, Inc., Sint-Martens-Latern, Belgium) was used to perform clustering and phylogenetic tree analyses. The allelic diversity of each VNTR loci (*h*) and the discriminating power according to the Hunter-Gaston index were calculated by using the algorithm shown in Figure 1.

Logistic regression analysis was done to compare MDR TB case-patients with pan-sensitive TB case-patients and to identify factors associated with clustering among MDR TB case-patients. We included the following variables in bivariate analysis: sex, age, occupation, history of close contact with a TB patient, health insurance, TB treatment history, symptom duration before first evaluation in SPTH, presence of cavity on chest radiograph, current alcohol use, current smoking, diagnostic sputum smear test results, HIV status, and Beijing genotype. Variables with a p value <0.2 were included in multivariable analyses. The final model was established by backward selection based on the fit of the model as tested with the likelihood ratio χ^2 test (p = 0.05). All statistical analyses were performed by using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA).

Ethical Issues

This research project was approved by the Chinese Ethical Committee for TB Operational Research, Chinese Center for Disease Control and Prevention, and SPTH. Patients were asked for written informed consent before drug susceptibility testing and genotyping were performed. Patients and TB hospitals were informed of test results, and the treatment regimen for each patient was adjusted according to the detected resistance profiles.

Results

Among the 1,530 *M. tuberculosis* isolates from all hospitalized patients during the study period, 556 (36.3%) were resistant to ≥1 first-line drugs (isoniazid, rifampin, streptomycin, or ethambutol), of which 313 (20.5%) were resistant to 1 drug, 143 (9.3%) were resistant to ≥1 drug but were not MDR (poly-drug resistant), and 100 (6.5%) were MDR. A total of 33 of the 100 MDR TB patients and 47 of the 97 patients with pan-sensitive TB were from Jinan city,

$$h = 1 - \sum x_i^2 \left[\frac{n}{n-1}\right]$$

$$\mathrm{HGI} = 1 - \left[\frac{1}{N(N-1)}\right] \sum_{J-1}^{s} n_j \left(n_j - 1\right)$$

Figure 1. The allelic diversity of each VNTR loci (*h*) and the discriminating power according to the Hunter-Gaston index (HGI) were calculated by using this algorithm (*32*). MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number of tandem repeats; x_i, the frequency of allele *i*; *n,* total no. of strains in the scheme; *N,* total number of strains in the typing scheme; *s*, total no. of different MIRU-VNTR patterns, n_j, no. of strains belonging to the j^{th} type.

the capital of Shandong Province; other patients were from other parts of Shandong Province. The 97 selected controls were comparable to all patients with pan-sensitive TB with regard to potential confounding variables, such as age (38 vs. 37 years, p = 0.79), sex (69.1% vs. 70.0% male, p = 0.83), and retreatment (7.2% vs. 7.6%, p = 0.89).

Genotyping Characteristics

The RD105 deletion test identified 94 of the 100 MDR TB strains and 77 (79%) of the 97 pan-sensitive strains as Beijing genotype strains ($\chi^2 = 9.19$, p = 0.002). Among 24 loci, MIRU-VNTR, QUB11b, Mtub21, and Mtub4 showed high allelic diversity ($h \geq 0.60$), and MIRU26, QUB26, MIRU31, MIRU10, Mtub39, QUB4156, MIRU39, and ETR-A showed middle allelic diversity ($h \geq 0.30$) (Table 1).

We identified 73 different MIRU-VNTR genotype profiles among the 100 MDR TB strains, comprising 59 unique strains and 41 strains in 14 clusters; thus, 41% of the MDR TB cases clustered. The number of isolates in DNA fingerprint clusters ranged from 2 to 6. The discriminating power according to the Hunter-Gaston index method was 0.989. Dendrograms of 100 MDR TB cases, which were analyzed by using the RD105 deletion test and the MIRU-VNTR method, are shown in Figure 2. No clustering was detected among non–Beijing genotype cases.

Determinants for Clustering among MDR TB Cases

In multivariate analysis comparing the 41 clustered MDR TB cases with the 59 nonclustered cases, we found that clustering was more likely to occur in isolates from patients who were ≤45 years of age (32 [50%] of 64) (adjusted odds ratio [OR] 3.5, 95% confidence interval [CI] 1.4–9.0), never previously treated (23 [51%] of 45; adjusted OR 2.6, 95% CI 1.1–6.2), and infected with Beijing genotype strains (41 of 100). Because the non–Beijing genotype strains did not include MDR TB strains, we did not calculate an OR.

Determinants for MDR TB

Sixty-one percent of MDR TB case-patients were male; 49% were <35 years of age, and 55% were receiving retreatment for TB (Table 2). In bivariate analysis, MDR TB was not significantly associated with current alcohol use, current smoking, diagnostic sputum smear test results, or HIV status (tested patients all had negative results). Occupation, health insurance, TB treatment history, symptom duration before first evaluation in SPTH, cavity on chest radiographs, and genotype strain were significantly associated with MDR TB (p<0.05) (Table 2). Several of these factors remained significant in multivariate analysis, including previous TB treatment (adjusted OR 12.0, 95% CI 4.5–31.8), history of TB symptoms for >3 months before first visit to SPTH (adjusted OR 3.0, 95% CI 1.0–9.2), and lack of health insurance (adjusted OR 2.4, 95% CI 1.1–5.1). MDR TB occurred less frequently in students (adjusted OR 0.2, 95% CI 0.1–0.7) than farmers. MDR TB cases were also more likely to be caused by a Beijing genotype TB strain (adjusted OR 4.3, 95% CI 1.4–13.9).

Table 1. Allelic diversity and number of repeats among 197 TB isolates, Shandong Province, People's Republic of China, April 2007–July 2009*

Locus†	Allelic diversity	Allele no.	No. repeats, range
QUB11b	0.703	6	1–7
Mtub21	0.642	7	1–7
Mtub4	0.621	5	1–5
MIRU26	0.595	8	3–11
QUB26	0.551	9	2–10
MIRU31	0.494	7	2–9
MIRU10	0.340	4	1–4
Mtub39	0.335	7	1–7
QUB4156	0.331	5	1–5
MIRU39	0.326	3	2–4
ETR-A	0.303	7	1–7
Mtub30	0.295	4	2–5
MIRU40	0.263	6	1–6
MIRU4	0.237	6	0–7
MIRU16	0.175	4	1–4
MIRU27	0.156	3	1–3
ETR-B	0.146	3	1–3
MIRU20	0.146	3	1–3
MIRU23	0.093	4	2–6
Mtub34	0.073	2	2–3
Mtub29	0.055	4	2–5
ETR-C	0.055	3	2–4
MIRU2	0.000	1	1
MIRU24	0.000	1	1

*Allelic diversity and number of repeats was determined by using a 24-locus mycobacterial interspersed repetitive unit (MIRU)–variable-number of tandem repeats. TB, tuberculosis.
†Loci are listed within each isolate group in descending order of allelic diversity.

Discussion

In this study in Shandong Province, the fact that 45% of new TB cases are MDR TB and the high percentage of clustering among isolates (51%) indicate substantial MDR TB transmission. The percentage of case clustering among persons previously treated for MDR TB was also substantial (33%). Thus, a large percentage of MDR TB cases in Shandong Province most likely were caused by recent transmission. Nosocomial transmission is not likely to explain the high percentage of clustering because sputum samples were obtained at hospital admission. MDR TB was more likely to be diagnosed in population groups of lower socioeconomic status, such as farmers, persons with low education level, and those without health insurance. These findings suggest that the prevalence of MDR TB among TB case-patients may be poverty related;

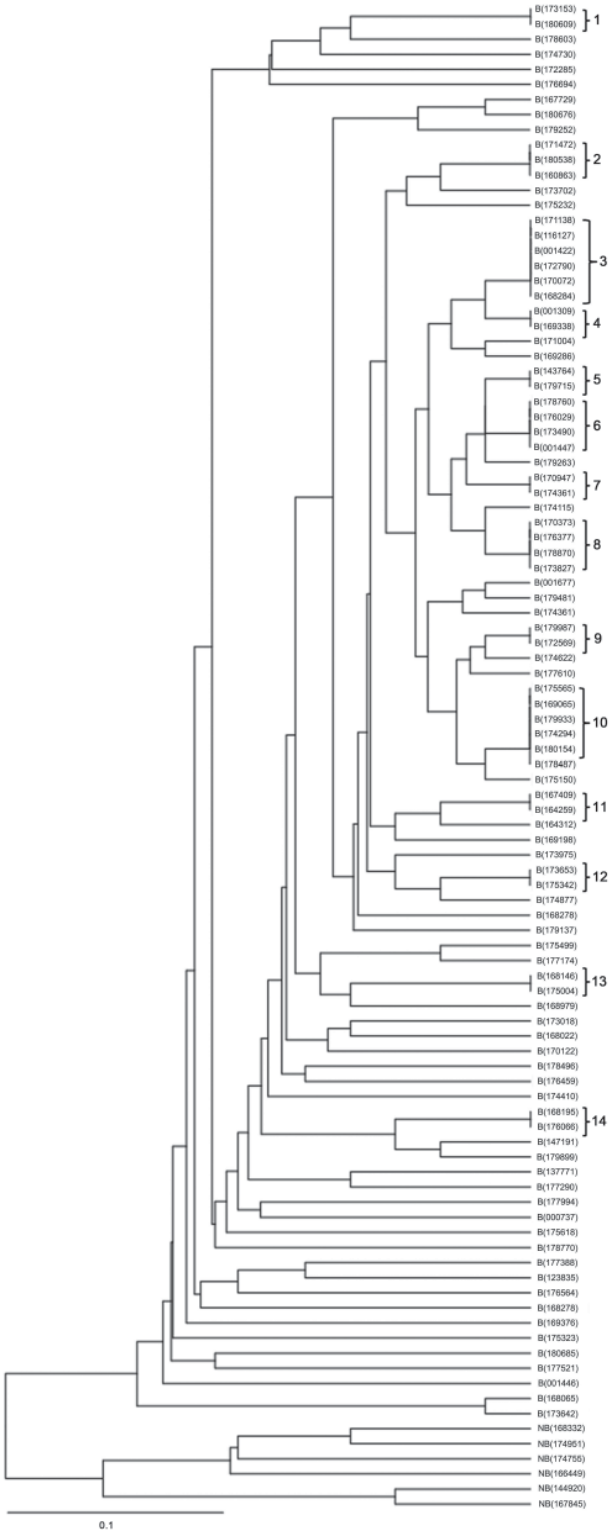

Figure 2. Dendrogram of 100 multidrug-resistant tuberculosis cases analyzed by using the RD105 deletion test and the MIRU-VNTR method. RD, region of difference; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number of tandem repeats; B, Beijing family; NB, no Beijing family. Scale bar indicates nucleotide substitutions per site.

therefore, these lower socioeconomic groups should be the highest priority for MDR TB prevention efforts.

Our molecular analysis found a high discriminative power for using 24-locus MIRU-VNTR genotyping. We found that 41% of MDR TB isolates shared identical VNTR profiles, and clusters of up to 6 cases in size were observed. In general, MDR TB has a low conversion rate and lengthy negative conversion period, given its high capacity for transmission (*9*). This phenomenon indicates that some MDR TB strains might give rise to widespread transmission of MDR TB.

Our genotyping analysis showed that strain clustering was more common among young patients, in new cases, and in TB caused by Beijing genotype strains. The finding regarding younger patients was in agreement with a previous study, suggesting that TB among young patients more frequently is clustered (*33*).

Most MDR TB cases occurred among patients who were previously treated, were less educated, lacked health insurance, or had a medical history of TB for ≥3 months before entering SPTH. Patients who have previously received treatment are at risk for MDR TB because of inadequate treatment of drug-sensitive TB or because of relapse after a previous episode of unrecognized MDR TB that was incorrectly treated as drug sensitive. Although reinfection also should always be considered (*34*), our findings reaffirm the need to prevent MDR TB through effective anti-TB drug regimens that are based on drug susceptibility profiles and are administered under direct supervision for the correct duration. Efforts are needed to strengthen basic TB diagnosis and treatment at the primary health care level, where such patients are likely to first contact the health system.

Beijing genotype strains are more common in Asia, former Soviet republics, and South Africa, but they are spreading worldwide (*35*,*36*). Several studies have shown that these strains are prevalent in China, especially northern China (*23*). A previous study found that non-Beijing strains represent 20.6% of strains in Shandong Province (*37*). In our sample from Shandong Province, Beijing genotype strains constituted 94% of MDR TB and 80% of pan-sensitive cases, suggesting that Beijing genotype is associated with MDR TB phenotype. Drobniewski et al. suggested that these Beijing strains have greater virulence and transmissibility and a tendency toward generation of MDR TB, although the mechanism remains unclear (*38*).

Our study has 2 main limitations. First, patients in this study were recruited only from 1 large TB hospital. The clustering proportion will depend on the completeness of the sampling, in which increasing sampling fractions will identify more clustering (*39*,*40*). Thus, our results likely underestimate the percentage of clustering. Because no reliable estimates exist of the incidence and prevalence

Table 2. Risk factors for MDR TB, Shandong Province, People's Republic of China, April 2007–July 2009*

Patient data	No. (%) patients with MDR TB, n = 100	No. (%) patients with pan-sensitive TB, n = 97	Crude OR (95% CI)	Adjusted OR† (95% CI)
Sex				
M	61 (61.0)	67 (69.1)	1	
F	39 (39.0)	30 (30.9)	0.7 (0.4–1.3)	
Age group, y				
15–34	49 (49.0)	56 (57.7)	1	
35–54	38 (38.0)	21 (21.6)	2.1 (1.1–4.0)	
≥55	13 (13.0)	20 (20.6)	0.7 (0.3–1.6)	
Occupation				
Farmer	39 (39.0)	29 (29.9)	1	1
Blue-collar worker	16 (16.0)	12 (12.4)	1.0 (0.4–2.4)	0.8 (0.2–2.4)
White-collar worker	37 (37.0)	39 (40.2)	0.7 (0.4–1.4)	0.6 (0.3–1.4)
Student	8 (8.0)	17 (17.5)	0.4 (0.1–0.9)	0.2 (0.1–0.7)
Close contact with TB patient				
No	90 (90.0)	90 (92.8)	1	
Yes	10 (10.0)	7 (7.2)	0.7 (0.3–1.9)	
Health insurance				
Yes	33 (33.0)	48 (49.5)	1	1
No	67 (67.0)	49 (50.5)	2.0 (1.1–3.5)	2.4 (1.1–5.1)
History of TB treatment				
New case	45 (45.0)	90 (92.8)	1	1
Retreatment case	55 (55.0)	7 (7.2)	15.7 (6.6–37.3)	12.0 (4.5–31.8)
Symptom duration before first evaluation in Shandong Provincial TB Hospital, mo				
<1	14 (14.0)	31 (32.0)	1	1
1–2	16 (16.0)	30 (30.9)	1.2 (0.5–2.8)	1.3 (0.5–3.8)
3–5	14 (14.0)	17 (17.5)	1.8 (0.7–4.7)	3.0 (1.0–9.2)
≥6	56 (56.0)	19 (19.6)	6.5 (2.9–14.8)	3.3 (1.2–9.1)
Cavity visible on radiograph				
No	55 (55.0)	67 (69.1)	1	
Yes	45 (45.0)	30 (30.9)	1.8 (1.0–3.3)	
Beijing genotype				
No	6 (6.0)	20 (20.6)	1	1
Yes	94 (94.0)	77 (79.4)	4.1 (1.6–10.6)	4.3 (1.4–13.9)

*MDR TB, multidrug-resistant tuberculosis; OR, odds ratio; CI, confidence interval.
†Adjusted for all other factors included in the multivariate model.

of MDR TB in Shandong Province, we do not know the percentage of MDR TB case-patients who were hospitalized in the study hospital and thus cannot estimate the extent to which we underestimated the clustering percentage. Studying a larger, geographically defined population will help us better understand the transmission dynamics of MDR TB and further identify populations or settings that should be made a priority for MDR TB prevention. Second, we did not investigate epidemiologic links between patients, such as home, job site, community, or congregate settings.

MDR TB presents one of the major challenges in TB control in China (*3*,*30*). Our findings of a relatively high prevalence of MDR TB among new TB cases and a high percentage of clustering among MDR TB cases show that MDR TB transmission is a large problem in Shandong Province, China, highlighting the risk for further occurrence of drug-resistant TB. The general programmatic management of MDR TB as well as specific measures are urgently needed to control transmission of MDR TB in China.

Our study highlights the challenges of controlling MDR TB in China. There is a trend toward lower risk for MDR TB in higher socioeconomic groups. The recent transmission of MDR TB may be contributing to a large percentage of cases. Without early diagnosis and effective treatment, these new cases will continue to generate further infections in the community. China must urgently scale up prevention of MDR TB through better infection control, enhancement of directly-observed treatments, universal use of appropriate diagnosis and treatment for MDR TB. Similarly, Beijing strains continue to predominate and to be associated with MDR TB, meaning that more dedicated research is needed to understand why these strains have survived and thrived in China, as well as other countries.

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Bacterial Causes of Empyema in Children, Australia, 2007–2009

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An increase in the incidence of empyema worldwide could be related to invasive pneumococcal disease caused by emergent nonvaccine replacement serotypes. To determine bacterial pathogens and pneumococcal serotypes that cause empyema in children in Australia, we conducted a 2-year study of 174 children with empyema. Blood and pleural fluid samples were cultured, and pleural fluid was tested by PCR. Thirty-two (21.0%) of 152 blood and 53 (33.1%) of 160 pleural fluid cultures were positive for bacteria; *Streptococcus pneumoniae* was the most common organism identified. PCR identified *S. pneumoniae* in 74 (51.7%) and other bacteria in 19 (13.1%) of 145 pleural fluid specimens. Of 53 samples in which *S. pneumoniae* serotypes were identified, 2 (3.8%) had vaccine-related serotypes and 51 (96.2%) had nonvaccine serotypes; 19A (n = 20; 36.4%), 3 (n = 18; 32.7%), and 1 (n = 8; 14.5%) were the most common. High proportions of nonvaccine serotypes suggest the need to broaden vaccine coverage.

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Empyema in children is a relatively uncommon disease that occurs in 0.7% of children with pneumonia (*1*). Many organisms cause empyema in children; *Streptococcus pneumoniae* is the most common (*2–6*). Other important causes, which are becoming increasingly frequent in several countries, are methicillin-sensitive *Staphylococcus aureus* (MSSA) (*2*,*7*,*8*) and methicillin-resistant *S. aureus* (MRSA). The latter is particularly problematic in indigenous communities (*9*). Other commonly identified organisms include *S. pyogenes, Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, and other *Streptococcus* spp. (*10*). The identification of causative organisms is usually determined by standard blood or pleural fluid cultures. Cultures are limited in that the yield can be as low as 8% (*11*), possibly because of prior antimicrobial drug treatment. Molecular techniques, such as PCR, are more sensitive in detecting causative organisms than standard culture (*11*) but are not routinely employed in laboratories for clinical use.

The 7-valent pneumococcal conjugate vaccine (PCV7) (Prevenar; Wyeth, Philadelphia, PA, USA) was introduced in Australia for immunocompromised and indigenous children <2 years of age in 2001 and was added to the national immunization schedule for all children <2 years in 2005 (www.medicareaustralia.gov.au/public/services/acir/index.jsp). Of >90 pneumococcal serotypes, the 7 included in the vaccine were responsible for 50%–70% of invasive pneumococcal disease (IPD) in children in most populations at the time of its development (*12*).

Many reports from around the world suggest an increase in the incidence of empyema in children (*1*,*6*,*13–19*). The reasons for this increase are unknown but may be related to IPD caused by emergent nonvaccine replacement serotypes, particularly serotypes 1, 3, and 19A after the introduction of PCV7 (*14–20*). However, this theory is

controversial because several studies identified an increase in empyema prevalence before the introduction of PCV7 (*5*,*21*,*22*). Because no Australian data exist on the bacterial causes of empyema, it is difficult to determine whether incidence in Australia is similar to reported trends in North America (*14*,*22*), the United Kingdom (*5*,*6*,*16*,*18*,*21*), Spain (*15*,*17*), and France (*13*).

The aims of this study were to identify the bacterial causes of empyema in children by using molecular techniques and to assess the efficacy of PCV7 by using molecular typing of invasive pneumococcal disease serotypes. This information may be helpful in deciding which of the newer conjugate pneumococcal vaccines should be introduced into national vaccination programs.

Methods

The Australian Research Network in Empyema was established in April 2007 and comprises all 13 major tertiary pediatric hospitals from all states and territories. Children with empyema were prospectively recruited over a 2-year period until April 2009.

Patients

A case of childhood empyema was defined by the principal site investigators as the presence of pus cells in the pleural fluid or bacteria isolated from the pleural fluid of a child with fever, respiratory symptoms, raised serologic inflammatory markers, and pleural fluid present on ultrasound image, chest radiograph, or computed tomography scan. Children with postoperative effusions were excluded.

Clinical data collected included age, sex, indigenous status, area of residence, risk factors, congenital or chromosomal abnormality, anatomic or functional asplenia, immunocompromise, and chronic illness. Vaccination status of recruited patients was obtained by either review of the child's hand-held health records (Blue Book), contacting the Australian Childhood Immunisation Registry (with parental permission) for patients <7 years of age (www.medicareaustralia.gov.au/public/services/acir/index.jsp), or, if these validated sources were unavailable, parental recall.

Microbiologic Investigations

Blood and pleural fluid specimens were cultured at local hospital microbiology laboratories by standard culture method. If growth was detected, Gram staining was performed and liquid media were subcultured onto horse blood agar. Isolates were identified by using conventional methods. Colonies resembling *S. pneumoniae* that contained gram-positive diplococci were identified by optochin- susceptibility and bile-solubility testing.

A separate aliquot of pleural fluid (in ideal circumstances, 10 mL) was collected, labeled according to the central coordinator's de-identification and specimen-tracking database, and stored at −20°C. Pleural fluid specimens were transported in batches on dry ice by a commercial transport company to the Centre for Infectious Disease and Microbiology Laboratory, Westmead Hospital, Westmead, New South Wales, Australia, for processing.

Streptococcus pneumoniae PCR

Total nucleic acid was extracted from pleural fluid specimens by using either NucliSENS easyMAG Total Nucleic Acid Extractor (bioMérieux Australia Pty Ltd, Sydney, NSW, Australia) with enzymes and lysis buffer provided, or SIGMA GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Sydney, NSW, Australia) with lysis buffer provided plus proteinase K, following the manufacturer's instructions.

S. pneumoniae PCR targeting the autolysin gene (*lytA*) was performed by using a TaqMan probe and primers as described by McAvin et al. (*23*), except that the result was read by spectrofluorometry and interpreted as described by Poddar et al. (*24*). Briefly, a 25-μL PCR containing 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 200 nmol/L of each primer, 120 nmol/L probe, 1.23U HotStar*Taq* DNA polymerase, and 10 μL of total nucleic acid yielded a 101-bp product. The PCR cycling conditions included initial denaturation at 95°C for 15 min, followed by 45 cycles at 96°C for 10 s, 63°C for 1 min, and a final extension step of 72°C for 2 min. The endpoint results were analyzed by calculating the postread to preread ratio. Samples with ratios of ≥2.78 were reported as positive and confirmed by using pulsed-field gel electrophoresis on a 2% gel, at 200 V for 40 min. Samples with ratios of ≤1.21 were reported as negative. The limit of detection of the assay was 6 CFU/mL.

Pneumococcal Serotype Identification

All samples in which *S. pneumoniae* was detected by PCR were examined by multiplex PCR reverse line blot (mPCR/reverse line blot [RLB]) to identify serotypes individually or in small groups of related serotypes (*25*,*26*). If serogroup 6 was identified, serotype-specific PCRs targeting *wciN* (to distinguish serotypes 6A and 6C) and the *wciP* single-nucleotide polymorphism, which distinguishes serotypes 6A and 6C from 6B (*27*), were performed. Samples that gave no signals in mPCR/RLB (result recorded as below detection level) and those in which only the *S. pneumonia*e positive control probes targeting *ply* or *lytA* produced signals (nontypeable) were further tested when sufficient DNA remained, by PCR and sequencing of the *cpsA-B* region of the capsular

polysaccharide synthesis (*cps*) gene cluster, as described and validated (*28*).

PCR for Other Pathogens

All pleural specimens were tested by separate in-house PCRs for the presence of *H. influenzae, M. pneumoniae*, *Chlamydia pneumoniae*, and *S. aureus* DNA. *M. pneumoniae* and *C. pneumoniae* PCR used fluorogenic probes with endpoint analysis in a fluorometer. Primers and probes were as follows: *C. pneumoniae* (target PstI fragment): primers Lab2f, 5′-GG AGA TAA AAT GGC TGG ACG-3′; Lab 2r, 5′-TAT GGC ATA TCC GCT TCG G-3′; probe Lab2p, 5′-6-FAM CAC GGA AAT AAA GGT GTT GTT TCC AAA ATCG-6-TAMRA-3′ (*29*); *M. pneumoniae* (target P1 protein gene): primers MYP-Fw, 5′-TCA GGT CAA TCT GGC GTG-3′; MYP-Rv, 5′-TCA AAC AGA TCG GCG ACT G-3′; probe MYP, 5′-(6-FAM) AGT TAC CAA GCA CGA GTG ACG GAA A-3′ (BHQ-1).

Conventional agarose gel electrophoresis was used for *H. influenzae* PCR. Primers used were Hinf OmP 6F, 5′-AAT GGT GCT GCT CAA ACT TT-3′; and Hinf Omp 6R, 5′-TCT AAG ATT TGA ACG TAT TCA CC-3′.

Testing for *S. aureus* DNA was undertaken by using a commercial multiplex-tandem PCR targeting the *S. aureus* nuclease gene *nuc,* and methicillin-resistance gene *mecA* (MRSA Easy-plex assay kit; AusDiagnostics, Sydney, New South Wales, Australia), as recommended by the manufacturer.

Assessment of Data Accuracy

To assess the completeness of case ascertainment, we contacted the coding departments of all participating hospitals at the end of the study and asked them to provide data on the number of children 0–18 years of age with empyema (classified according to International Classification of Diseases [ICD] codes J86.0 [pyothorax with fistula] or J86.9 [pyothorax without fistula]) who were discharged from each hospital from April 1, 2008, through April 30, 2009. This period was chosen because it represented a time when all hospitals were actively recruiting. These data were compared with our own.

Descriptive statistics were used for all analyses. No power calculation was required because this was an epidemiologic study aiming to capture all cases of empyema.

This study was approved by the local human research ethics committee at each site, and registered with The Australian and New Zealand Clinical Trial Registry (ACTRN12607000476437). Informed parental consent was obtained for each patient before blood and pleural fluid samples were collected.

Results

A total of 174 children were recruited over a 2-year period from April 2007 through April 2009, with a median recruitment rate of 6 (range 0–19) per month. Over a 1-year period, study sites recruited a median of 51.5% (range 0%–200%) of actual admissions identified by ICD coding discharge summaries. Demographic information was available from 172 patients (Table 1); of these, 70 (40.7%) were fully vaccinated (median age 2.1 years, range 0.4–5.3 years); 18 (10.5%) were partially vaccinated (median age 4.2 years, range 0.6–5.4 years); 56 (32.6%) were not vaccinated (median age 7.7 years, range 1–15.5 years); and 28 (16.3%) had no record of vaccination status (median age 6.1 years, range 0.6–14.4 years).

Of the 174 children recruited, culture results were available for 172; 140 had blood and pleural fluid cultured, 20 had only pleural fluid cultured, and 12 had only blood cultured. Of 152 blood and 160 pleural fluid cultures, 120 (78.9%) and 107 (66.9%), respectively, were negative. The bacteria isolated are shown in Table 2.

PCRs for *S. pneumoniae*, *H. influenzae*, *M. pneumoniae*, *C. pneumoniae*, and *S. aureus* were performed on 145 (83.3%) pleural fluid specimens (Figure 1). One or more of these organisms was detected by PCR in 88 specimens: *S. pneumoniae,* 74 (51.0%); *S. aureus*, 13 (9.0%; 1 with *S. pneumoniae*); *H. influenzae*, 4 (2.8%; 3 with *S. pneumoniae*); *M. pneumoniae*, 1 (0.7%); *C. pneumoniae*, 1 (0.7%; with *S. pneumoniae*). Of the 13 *S. aureus*–positive specimens, 6 were MSSA and 7 MRSA.

Table 1. Characteristics of 172 children with empyema, Australia, 2007–2009*

Characteristic	Value
Median age, y (range)	3.9 (0.4–15.5)
Sex	
M	93 (54.1)
F	79 (45.9)
Indigenous status	
Aboriginal	8 (4.7)
Other	160 (93.0)
Not recorded	4 (2.3)
State/territory	
Queensland	59 (34.3)
New South Wales	52 (30.2)
Victoria	37 (21.5)
Western Australia	16 (9.3)
South Australia	4 (2.3)
Tasmania	2 (1.2)
Australian Capital Territory	2 (1.2)
Northern Territory	0
Chronic respiratory diseases	7 (4.0)
Congenital diseases	3 (1.7)
Potentially immunocompromised	6 (5.5)
Cardiac disease	1 (0.6)

*Values are no. (%) unless otherwise indicated.

Table 2. Bacteria isolated by culture of blood and pleural fluid samples and by PCR of pleural fluid samples from children with empyema, Australia, 2007–2009*

Organism	No. (%) positive samples		
	Blood culture, n = 152	Pleural fluid	
		Culture, n = 160	PCR, n = 145
Streptococcus pneumoniae	19 (12.5)	12 (7.5)	74 (51)
S. pyogenes	3 (2.0)	14 (8.8)	NA
S. milleri	NA	4 (2.5)	NA
MSSA	1 (0.7)	11 (6.8)	6 (4.1)
MRSA	1 (0.7)	6 (3.8)	7 (4.8)
Coagulase-negative staphylococci	4 (2.6)	2 (1.3)	NA
Haemophilus influenzae	1 (0.7)	NA	4 (2.8)
Mycobacterium tuberculosis	NA	1 (0.6	NA
Pseudomonas aeruginosa	NA	1 (0.6)	NA
Mycoplasma pneumoniae	NA	NA	1 (0.7)
Chlamydia pneumoniae	NA	NA	1 (0.7)
Other†	4 (2.6)	4 (2.5)	NA

*NA, not applicable; MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *S.s aureus*.
†Blood cultures: 1 isolate each of *Streptococcus sanguinis; Staphylococcus hominis* (from a specimen in which *S. aureus* was also isolated); *Neisseria meningitidis*; and *Actinomyces naeslundii.* Pleural fluid: 1 isolate each of *Streptococcus oralis; Staphylococcus cohni; Eikenella corrodens;* and *Bacteroides fragilis* (the last 2 from the same specimen, from which *S. milleri* was also isolated*).* Both MRSA and MSSA were cultured from 1 pleural fluid specimen.

Pneumococcal serotypes were identified in 52 (70.3%) of 74 *S. pneumoniae* PCR-positive specimens; 3 specimens contained 2 serotypes (Table 3). Sufficient DNA could not be obtained for 22 *S. pneumoniae*–positive specimens to identify serotypes, including 14 in which there was no signal in mPCR/RLB and 8 in which 1 or both *S. pneumoniae*–specific (*ply* and/or *lytA*) probe signals, but none of the serotype-specific probes, were positive on RLB.

Vaccination status was available for 45 (86.5%) of 52 children who had a pneumococcal serotype detected on PCR. The effect of vaccination status on the acquisition of specific serotypes, in relation to age and vaccination status, was assessed (Figure 2).

Discussion

This study supports previous reports from different countries that have identified *S. pneumoniae*, *S. aureus,* and *S. pyogenes* as notable causes of childhood empyema (*2*–*4*,*8*,*17*,*21*). Most (96.4%) of identified pneumococcal serotypes were nonvaccine related, reflecting the effectiveness of the PCV7. Furthermore, this study highlights that PCR is more sensitive than culture for identifying pathogens.

Data on bacterial causes of childhood pneumonia in this geographic region are lacking (*30*). Before this study, the most comprehensive data relating to pneumococcal serotypes causing disease in children were from routine typing of sterile site isolates from cases of IPD reported to the Nationally Notifiable Diseases Surveillance System. However, surveillance of IPD does not specifically report on empyema.

By far, the most common organism identified was *S. pneumoniae*. A variety of organisms other than *S. pneumoniae* were detected by culture and PCR. After *S. pneumoniae*, *S. aureus* was the next most common pathogen, which was identified by culture in 17 pleural fluid and 2 blood specimens (12 MSSA, 7 MRSA) and by PCR in 13 specimens (Table 2). The occurrence of MRSA as a cause of community-acquired pneumonia and empyema is of particular concern because it is associated with more severe disease and a higher rate of complications than MSSA (*9*).

H. influenzae was detected by PCR in 4 children, 3 of whom also had positive PCR results for *S. pneumoniae*. *S. pyogenes*, *Mycobacterium tuberculosis,* and *P. aeruginosa* were isolated only by culture (individual PCR assays for these organisms were not available), and *M. pneumoniae* was detected by PCR in 1 child (Figure 1). All of these organisms are recognized causes of empyema in children (*10*). Although *C. pneumoniae* is a recognized cause of lower respiratory tract infection in children (*31*,*32*), its contribution to empyema has not been investigated previously. It was detected by PCR in only 1 child, with *S. pneumoniae* in the same specimen, which suggests that *C. pneumoniae* is not a major cause of empyema in children.

This study confirmed the findings of others, demonstrating enhanced sensitivity of molecular techniques (*11*,*33*–*35*). PCR detected many more *S. pneumoniae* isolates in pleural fluid than in cultures (51.0% vs. 7.5%), and thus routine use of PCR-based serotype identification in children with empyema may improve the accuracy of pneumococcal disease surveillance, which is

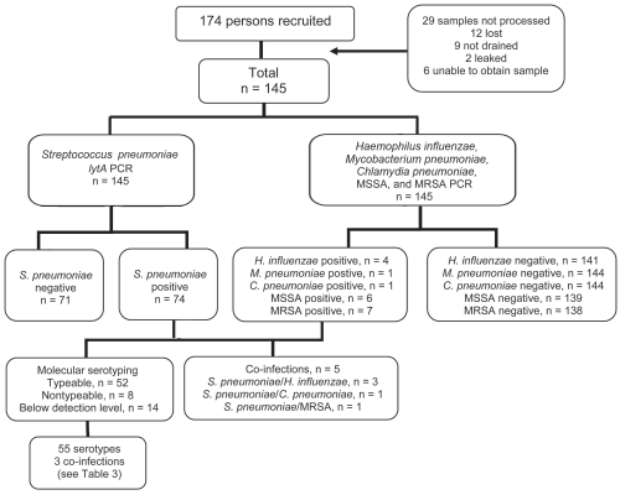

Figure 1. Flow diagram for PCR testing for bacterial pathogens in samples from children with empyema, Australia, 2007–2009. MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*.

Table 3. *Streptococcus pneumoniae* serotypes identified in 52 PCR-positive specimens from children with empyema, Australia, 2007–2009*

Serotype	No. (%) specimens
PCV7 serotypes	2
14	1 (1.8)
9V/9A	1 (1.8)
Nonvaccine serotypes	53
19A	20 (36.4)
3	18 (32.7)
1	8 (14.5)
7F/7A	2 (3.6)
22F/22A	2 (3.6)
6C	1(1.8)
15F	1 (1.8)
21	1 (1.8)

*Two serotypes were identified in 3 specimens: 19A/3, 19A/1, and 6C/15F. The multiplex PCR reverse line blot assay cannot distinguish some pairs or groups of closely related serotypes, including 7F/7A, 22F/22A, and serogroup 6. Individual serotypes within serogroup 6 were distinguished by using serotype-specific PCR. PCV-7, 7-valent pneumococcal conjugate vaccine.

essential for development of new vaccines with broader range of pneumococcal serotypes. In contrast, however, for 4 patients who had a culture positive for *S. aureus*, PCR results were negative. We are unclear why this occurred, but this PCR was performed last in the sequence, and results may have been due to insufficient DNA. Although PCR did not increase the yield of *S. aureus*, it can detect it more rapidly than culture, enabling rapid change to appropriate therapy, especially when MRSA is found.

Reports on childhood empyema pneumococcal serotype distribution from Europe and the United States show differences. Studies in Spain (*8*,*17*), the United Kingdom (*16*,*21*,*36*), and the United States (*14*) have reported a predominance of serotype 1, while in other US studies, 19A is the most common serotype (*20*,*22*); both are non–PCV7 serotypes. Bekri et al. (*13*) identified serotypes 1 and 19A as emerging serotypes in France and also showed that serotype 1 was predominant in children >5 years of age; serotype 19A appeared to only affect children <5 years of age. This age distribution was similarly reported in another study (*37*). The serotype distribution in our study was similar; serotypes 1, 3, and 19A were predominant (Figure 2), and most serotypes 3 and 19A were identified in children <5 years of age, similar to results previously reported (*37*). Although serotype 1 infections were identified across all age groups, most were in children >5 years of age.

We were reassured that only 2 children in our study had serotypes covered by PCV7; 1 child was covered partially, and the other had not been vaccinated. Overall, this study suggests the efficacy of the PCV7, as previously confirmed (*38*). However, public health authorities should be concerned that most pneumococcal infections were caused by nonvaccine serotypes, possibly related to replacement disease after the introduction of PCV7 onto the national vaccination schedule in 2005. We do not have serotype data specific to empyema prior to 2005, but this has occurred in other countries and affects all IPD, including meningitis (*39*). Most studies that compare pre– and post–pneumococcal vaccine effects have shown near extinction of PCV7 serotypes, along with dramatic increases in nonvaccine serotypes, predominantly 1, 3, and 19A (*14*,*15*,*17*,*20*).

Although the reasons behind serotype changes have not been determined fully, ongoing enhanced surveillance may help clarify them over time, enabling us to predict future serotype trends and tailor new vaccines accordingly. This ability is particularly relevant as 2 new vaccines with broader coverage of pneumococcal serotypes—10-valent pneumococcal conjugate vaccine, with additional serotypes 1, 5, and 7F, and 13-valent pneumococcal conjugate vaccine, with additional serotypes 1, 3, 5, 6A, 19A, and 7F—are being are being added to national vaccination schedules. The 10-valent vaccine offers protection against nontypeable *H. influenzae* through the use of an *H. influenzae* conjugate protein.

Our study has several limitations, nevertheless. First, we cannot know whether the number of children recruited in this study is an accurate snapshot of Australia's true empyema prevalence in children. However, after comparing ICD empyema codes with study recruitment rates over a 1-year period, we determined that we recruited a median of 51.5% (range 0%–200%) of empyema patients admitted to all major pediatric tertiary hospitals recorded by ICD. A limitation of this approach is that we were not able to verify the coding accuracy in each of the 13 hospitals. One likely reason why we did not capture all the cases may be because some patients received treatment from physicians at participating centers who were unaware of the study. Also, some children may have received treatment at

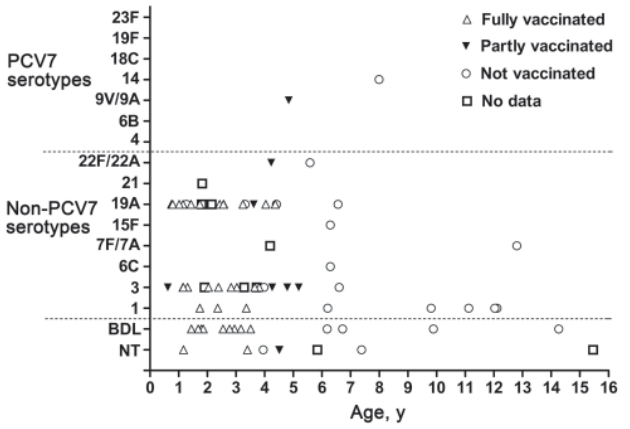

Figure 2. *Streptococcus pneumoniae* serotype distribution in relation to age and vaccination status of children with empyema, Australia, 2007–2009. PCV7, 7-valent pneumococcal conjugate vaccine.

smaller rural hospitals where the study was not conducted, even though we have recently shown that most patients are treated in 1 of the tertiary pediatric hospitals included in this study (*1*). We recruited patients from all states and territories in Australia, which is the strength of the study. A limitation of the PCR data is that the bacteria assessed were restricted to *H. influenzae, M. pneumoniae*, *C. pneumoniae,* and *S. aureu*s. They are all potentially important bacterial pathogens in empyema in children, however (*10*). The use of broader PCR, such as 16sPCR, may have detected more organisms, but it is an expensive test and our previous experience suggests that the yield of notable pathogens is poor and that positive results often reflect contamination.

In conclusion, we have demonstrated a wide variety of bacterial causes for empyema in children. Most infections were caused by non–PCV7 pneumococcal serotypes, which suggests that coverage of pneumococcal serotypes by vaccines needs to be broadened. Ongoing enhanced molecular surveillance is required, particularly to assess the effects of newer vaccines, such as 10-valent and 13-valent pneumococcal conjugate vaccines.

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Clinical Implications of Azole Resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009

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Learning Objectives

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

- Describe the prevalence of itraconazole resistance in clinical *A. fumigatus* isolates on the basis of a prospective, nationwide, multicenter surveillance study in the Netherlands
- Describe risk factors for development of itraconazole resistance in *A. fumigatus* isolates on the basis of that study
- Describe outcomes associated with development of itraconazole resistance in *A. fumigatus* isolates on the basis of that study

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The prevalence and spread of azole resistance in clinical *Aspergillus fumigatus* isolates in the Netherlands are currently unknown. Therefore, we performed a prospective nationwide multicenter surveillance study to determine the effects of resistance on patient management strategies and public health. From June 2007 through January 2009, all clinical *Aspergillus* spp. isolates were screened for itraconazole resistance. In total, 2,062 isolates from 1,385 patients were screened; the prevalence of itraconazole resistance in *A. fumigatus* in our patient cohort was 5.3% (range 0.8%–9.5%). Patients with a hematologic or oncologic disease were more likely to harbor an azole-resistant isolate than were other patient groups ($p<0.05$). Most patients (64.0%) from whom a resistant isolate was identified were azole naive, and the case-fatality rate of patients with azole-resistant invasive aspergillosis was 88.0%. Our study found that multiazole resistance in *A. fumigatus* is widespread in the Netherlands and is associated with a high death rate among patients with invasive aspergillosis.

Azoles are the primary class of antifungal agents used for evidence-based treatment and prevention of *Aspergillus* spp. diseases (*1*). Azoles are the only class of mold-active agents that can be used orally, and voriconazole and posaconazole have improved the survival of patients at risk for invasive aspergillosis (*2–5*). However, recent reports describe the emergence of acquired resistance of *Aspergillus* spp. to azole compounds (*6–16*). Azole resistance may develop in patients with cavitary lung lesions, such as aspergilloma, or in patients with allergic bronchopulmonary aspergillosis who are treated with mold-active azoles, most notably, itraconazole (*7*,*17*). In these patients, resistance is most commonly observed in *Aspergillus fumigatus*, and the isolates may be resistant to only itraconazole (ITZ) or exhibit a multi-azole– or pan-azole–resistant phenotype. The phenotype depends on the underlying resistance mechanism, which commonly involves point mutations in the *cyp51A*-gene, the target for antifungal azoles (*6–8*,*10*,*11*,*17–21*). A wide range of mutations was found in azole-resistant *Aspergillus* spp. isolates that were cultured from clinical samples from patients treated with azoles, and individual azole-resistant *Aspergillus* spp. colonies harbored different resistance mechanisms (*7*).

In the Netherlands, a highly dominant resistance mechanism was found in isolates from epidemiologically unrelated patients (*6*,*9*). The dominant resistance mechanism consisted of a substitution of leucine for histidine at codon 98 of the *cyp51A*-gene in combination with a 34-bp tandem repeat in the promoter region of this gene (TR/L98H) (*6*,*11*). The corresponding phenotype showed resistance to itraconazole and intermediate susceptibility or resistance to voriconazole, posaconazole, or both (*6*,*20*). TR/L98H isolates were recovered from azole-treated and azole-naive patients with *Aspergillus* spp. diseases (*6*,*9*,*10*,*22–24*). Azole resistance may be associated with a high probability of azole treatment failure, a possibility that is supported by preclinical evidence (*7*,*9*,*22–26*).

On the basis of the above-mentioned observations, we hypothesized that resistance might have emerged as a consequence of exposure of *A. fumigatus* to azole fungicides in the environment (*27*). Preliminary studies indeed showed that *A. fumigatus* isolates harboring TR/L98H could be recovered from the environment (*28*,*29*). Furthermore, TR/L98H isolates were cross-resistant to certain azole fungicides (*27*,*28*).

The spread and prevalence of azole resistance in clinical *A. fumigatus* isolates are unknown. Various studies in the Netherlands that have analyzed *Aspergillus* spp. culture collections indicate that the prevalence ranges from 1.8% to 12.8% (*6*,*30*,*31*). Obtaining insight into the spread of azole resistance is essential for determining the implications of resistance for patient management strategies and public health. We therefore conducted a prospective surveillance study in 7 university medical centers in the Netherlands.

Methods

Study Design

All *Aspergillus* spp. isolates cultured from clinical samples that were processed in the 7 university medical microbiology laboratories were routinely screened for the presence of azole resistance, irrespective of the clinical relevance of the culture result. The University Medical Centers are located in 7 different cities in the Netherlands, and clinical isolates were screened during a 20-month period (June 2007–January 2009). Patient data were recorded in a Web-based database. An online questionnaire was completed for every collected isolate. The questionnaire included questions about isolate characteristics (species identification and date of isolation) and patient characteristics (age, sex, and underlying disease).

Screening ITZ Agar Slants

Aspergillus spp. colonies that grew in primary cultures were subcultured on Sabouraud agar slants supplemented with 4 mg/L of ITZ and incubated at 35°C–37°C. The colonies' ability to grow on the ITZ agar slants was assessed after 48 hours of incubation. For every isolate that was able to grow on the ITZ agar slants (ITZ positive), the primary culture isolate was sent to the Radboud University Nijmegen Medical Center for further analysis. Isolates that failed to grow on the ITZ agar slants (ITZ negative) were not analyzed. However, for all isolates that were screened, the online questionnaire was completed. At the screening sites, the *Aspergillus* spp. isolates were identified to species level by conventional methods, i.e., thermotolerance and

macroscopic and microscopic assessment of culture morphologic features.

Proficiency Testing

The ITZ agar slants were prepared at the Radboud University Nijmegen Medical Center and distributed to the other medical microbiology laboratories. Each center was provided with a protocol and a set of 6 *A. fumigatus* isolates. Four isolates were resistant to ITZ (MIC >16 mg/L), while 2 were susceptible (ITZ MICs 0.125 and 0.25 mg/L). The centers were blinded for the resistance profiles and were asked to determine the ability of the isolates to grow on the ITZ agar slants.

Analysis of ITZ-positive Isolates

Every ITZ-positive isolate was analyzed for certain phenotypic and genotypic features. The phenotypic analysis included the morphologic features of the isolate and susceptibility testing according to established standards (*32*) by using a broth microdilution format. MICs were determined for ITZ, voriconazole, and posaconazole. Genotypic analyses were performed by complete sequencing of the *cyp51A* gene by using the reference sequence of strain no. AF338659 from GenBank.

For the confirmed ITZ-positive isolates, additional patient data that included azole exposure <3 months before the date of isolation of the resistant isolate and the presence of *Aspergillus* spp. disease were requested from the treating physician. Patients with cancer and invasive aspergillosis were classified according to the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group definitions (*33*). In addition, patient data were collected on treatment and outcome 3 months after diagnosis.

Statistical Analysis

Statistical analyses were performed by using SPSS version 17.0 (IBM, Somers, NY, USA). Analyses consisted of χ^2 tests and a calculation of the κ coefficient.

Results

Proficiency Testing

Four of 6 control *A. fumigatus* isolates were resistant to ITZ and expected to grow on the ITZ agar slants, while the remaining 2 isolates were ITZ susceptible and should not grow on the ITZ agar slants. The 4 isolates with the azole-resistant phenotype harbored the TR/L98H resistance mechanism. For 3 ITZ-positive and the 2 ITZ-negative isolates, 100% agreement was found between the 7 centers. For 1 ITZ-positive isolate, the assessment of growth on the ITZ agar slant was incorrect from 3 centers; this isolate was a slowly sporulating *A. fumigatus* isolate. The κ coefficient calculated to assess the reproducibility of the method was 0.81 (*34*).

Mycology

During June 2007 through January 2009, we screened 2,062 *Aspergillus* spp. isolates from 1,385 patients for azole resistance using the ITZ agar slants. Most isolates were identified as *A. fumigatus* (1,792/2,062 [86.9%]) (Table 1). For 50 (2.5%) *Aspergillus* spp. isolates, species identification was not done. Most *Aspergillus* spp. isolates were isolated from respiratory samples; 1,461 of 2,062 (70.9%) were from sputum cultures, and 60 (2.9%) were from cultures derived from clinical specimens obtained from sterile sites (i.e., tissue specimens obtained through invasive procedures or at autopsy) (Table 1).

Ninety isolates were able to grow on the ITZ agar slants and were sent to the Radboud University Nijmegen Medical Center for further analyses. In vitro susceptibility testing showed that for 84 (93.3%) of 90 ITZ-positive isolates, the MIC of ITZ was >2 mg/L, which was considered resistant (*20*). Most ITZ-positive isolates also exhibited non–wild-type susceptibility profiles to voriconazole and posaconazole. A resistant phenotype for voriconazole (>2 mg/L) and posaconazole (>0.5 mg/L) was observed in 67/84 (79.8%) and 14/84 (16.7%) of ITZ-positive isolates, respectively. An intermediate susceptibility profile (2 mg/L for voriconazole and 0.5 mg/L for posaconazole) was observed in 12/84 (14.3%)

Table 1. Characteristics of screened susceptible and resistant isolates of *Aspergillus* spp., the Netherlands, 2007–2009*

Source and species	No. (%) susceptible, n = 1,978	No. (%) resistant, n = 84
Specimen source		
Sputum	1,397 (70.6)	64 (76.2)
Ear swab	176 (8.9)	3 (3.6)
BAL fluid	97 (4.9)	6 (7.1)
Bronchus secretion	66 (3.3)	2 (2.4)
Throat/nasal swab	66 (3.3)	1 (1.2)
Tissue	55 (2.8)	5 (6.0)
Skin swab/nail	38 (1.9)	1 (1.2)
Mouth wash	26 (1.3)	1 (1.2)
Pus/wound swab	16 (0.8)	1 (1.2)
Bronchial wash	11 (0.6)	0
Feces	8 (0.4)	0
Unknown	22 (1.1)	0
Species		
A. fumigatus	1,710 (86.5)	82 (97.6)
A. flavus	98 (5.0)	0
A. niger	52 (2.6)	2 (2.4)
A. terreus	35 (1.8)	0
A. nidulans	14 (0.7)	0
A. versicolor	13 (0.7)	0
A. glaucus	6 (0.3)	0
Unknown	50 (2.5)	0

*BAL, bronchoalveolar lavage.

Table 2. Characteristics of itraconazole-positive *Aspergillus fumigatus* isolates, the Netherlands, 2007–2009*

No. isolates	Mutations in the *Cyp51A* gene	Median MIC, mg/L (range)		
		Itraconazole	Voriconazole	Posaconazole
74	TR/L98H†	>16 (16–>16)	8 (1–16)	0.5 (0.25–2)
1	G54W†	>16	0.5	>16
1	P216L†	16	2	0.5
1	F219I†	>16	0.25	0.25
1	Series‡	>16	>16	1
4	None	>16 (16–>16)	4 (0.5–4)	0.2 (0.125–1)

*In vitro susceptibility testing was performed according to the Clinical and Laboratory Standards Institute M38A2 method (*32*).
†Mutations that have previously been shown to be associated with azole resistance in *A. fumigatus* (*7*,*20*,*21*).
‡Series of mutations in *Cyp51A*-gene: F46Y, G89G, M172V, N248T, D255E, L358L, E427K, and C454C (*8*).

and 44/84 (52.4%) of ITZ-positive isolates, respectively (Table 2) (*20*).

Sequence-based identification classified 82 ITZ-positive isolates as *A. fumigatus* and 2 as *A. niger*. Sequencing of the *cyp51A* gene showed a substitution of leucine for histidine at codon 98 in combination with a 34-bp tandem repeat in the gene promoter in 74 (90.2%) of 82 resistant *A. fumigatus* isolates (Table 2). Other *cyp51A* mutations were found in 3 isolates (substitutions G54W, P216L, and F219I) (Table 2) (*7*,*8*,*20*,*21*). In 1 isolate, a series of mutations was found, and in the remaining 4 azole-resistant *A. fumigatus* isolates (4.9%), no mutations were found in the *cyp51A* gene (*8*). Because *A. niger* has no known resistance mechanisms, the 2 azole-resistant *A. niger* isolates were not further analyzed.

Prevalence of Azole Resistance

A median of 100 isolates were screened each month, with a range of 78–140 isolates per month. The total number of screened clinical isolates per center ranged from 130 to 449 and were recovered from 84 to 238 patients. The collection of isolates over the study period and the distribution of the recovery of resistant isolates are shown in Figure 1. Overall, 82 (4.6%) of 1,792 screened *A. fumigatus* isolates were azole resistant. The per-patient analysis showed a prevalence of 5.3%. Figure 2 shows the prevalence of resistance in *A. fumigatus* per center. The prevalence of azole resistance in *A. niger* was 3.8%. No seasonal variation was observed.

Patient Characteristics

The screened *A. fumigatus* isolates were cultured from 1,192 patients, while other *Aspergillus* spp. isolates were recovered from 193 patients. Among the patients who harbored *A. fumigatus* isolates, the predominant underlying diseases were cystic fibrosis (382/1,192 [32.1%]) and other pulmonary diseases (265/1,192 [22.2%]) Almost 12% (138/1,192) of the *A. fumigatus* isolates were from patients who had hematologic/oncologic diseases. The distribution of underlying diseases is shown in Table 3.

Characteristics of Patients with Azole-Resistant Isolates

The 82 confirmed ITZ-resistant *A. fumigatus* isolates were recovered from 63 patients. The patients' ages ranged from 1 to 85 years, with a mean age of 48.9 years. The sex distribution was equal.

Overall, the distribution of underlying diseases in patients with an ITZ-resistant isolate was similar to that observed in the group with ITZ-susceptible isolates, with 1 exception. In the group of patients with ITZ-resistant isolates, significantly more patients had hematologic/

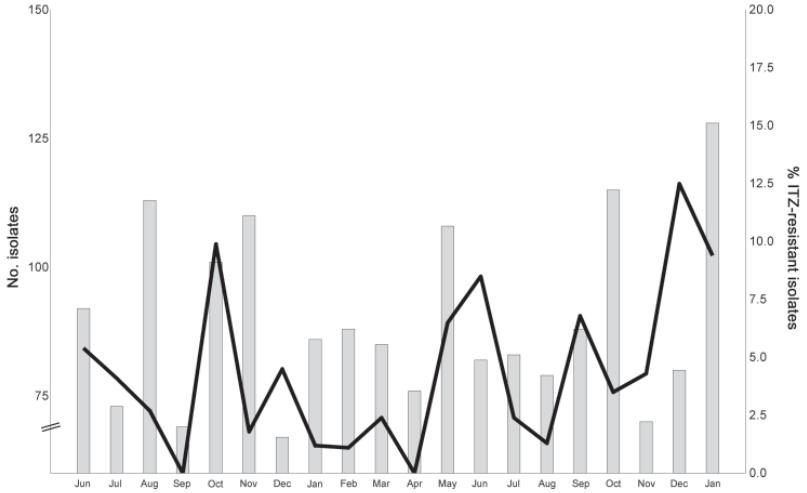

Figure 1. Number of screened *Aspergillus* spp. isolates per month (bars) and prevalence (%) of azole resistance (line), the Netherlands, 2007–2009. ITZ, itraconazole.

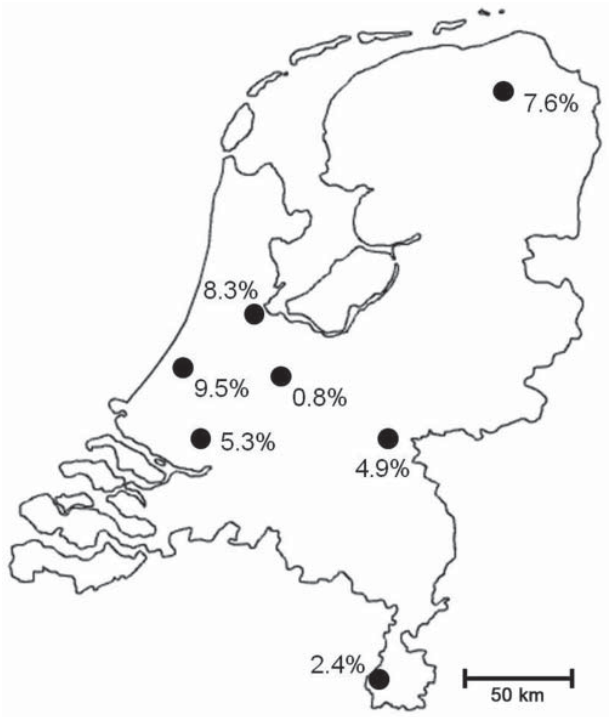

Figure 2. Prevalence (%) of azole-resistant *Aspergillus fumigatus* infections in university medical centers, the Netherlands, 2007–2009.

oncologic diseases than in the group that harbored ITZ-susceptible isolates (13/63 [20.6%] and 125/1,129 [11.1%], respectively; p<0.05; Table 3).

Aspergillus disease caused by an ITZ-resistant isolate was diagnosed in 23 patients (36%). Invasive aspergillosis was diagnosed in 8 patients (13%), 6 with confirmed and 2 with probable cases (Table 4). Four of these patients were azole naive, and 1 patient had been previously treated with fluconazole, which has no activity against *Aspergillus* species. All *A. fumigatus* isolates from patients with azole-resistant invasive aspergillosis harbored the TR/L98H resistance mechanism (Table 4). All 5 patients who were treated with voriconazole monotherapy died within 3 months of receiving a positive culture result. For 3 patients, voriconazole therapy was switched to another class of antifungal compounds, i.e., echinocandin, polyene, or both, but only 1 of those 3 patients survived. Overall, 7 (87.5%) of 8 patients with azole-resistant aspergillosis died within 3 months (Table 4).

Noninvasive *Aspergillus* spp. disease with an ITZ-resistant isolate was diagnosed in 15 patients. Five patients had cystic fibrosis and allergic bronchopulmonary aspergillosis. Six patients had an aspergilloma; of these patients, 2 were immunocompromised, 1 because of AIDS and 1 because of Job syndrome. Three patients sought treatment for otomycosis; 1 patient had *Aspergillus* spp. sinusitis. Data on previous exposure to azoles were available for 14 patients with noninvasive *Aspergillus* spp. disease; and 9 (64.2%) of these patients were azole naive. The TR/L98H mutation was the dominant resistance mechanism, because it was present in 12 (80%) of 15 isolates. Overall, 14 (64.3%) of 22 patients with azole-resistant *Aspergillus* spp. disease and known status of azole exposure were azole naive at the time the resistant isolate was cultured.

Discussion

Azole resistance is widespread in clinical *A. fumigatus* isolates in the Netherlands, and resistant isolates were found at all participating university medical centers. The overall prevalence was 5.3%, which is in a similar range as observed in the fungus culture collection of the Radboud University Nijmegen Medical Center (*6*). However, the prevalence varied widely between the centers, with 1 center

Table 3. Underlying diseases of patients from whom azole-susceptible and -resistant *Aspergillus fumigatus* isolates were recovered, the Netherlands, 2007–2009

Underlying condition	No. (%) patients with susceptible isolates, n = 1,129	No. (%) patients with resistant isolates, n = 63	p value*
Cystic fibrosis	365 (32.3)	17 (27.0)	0.38
Pulmonary disease, excluding cystic fibrosis	251 (22.2)	14 (22.2)	1.00
Hematologic/oncologic disease	125 (11.1)	13 (20.6)	0.02
Otorhinolaryngologic disease	63 (5.6)	3 (4.8)	0.78
Internal disease	85 (7.5)	8 (12.7)	0.14
Solid organ transplantation	26 (2.3)	2 (3.2)	0.66
Intensive care unit patient	31 (2.7)	2 (3.2)	0.84
Cardiac disease	9 (0.8)	1 (1.6)	0.50
Chronic granulomatous disease	5 (0.4)	0	0.60
Postoperative condition	23 (2.0)	1 (1.6)	0.81
Neurologic disease	2 (0.2)	0	0.74
Disease in children, not specified	4 (0.4)	0	0.64
Dermatologic disease	6 (0.5)	1 (1.6)	0.29
Other	91 (8.1)	1 (1.6)	
Unknown	43 (3.8)	0	

*χ^2 test.

Table 4. Characteristics of patients with azole-resistant invasive aspergillosis, the Netherlands, 2007–2009*

Patient age, y/ sex	Underlying disease	Disease	No. positive cultures†	Resistance mechanism	VCZ MIC, mg/L	Prior azole treatment (duration)‡	Treatment§	Outcome at 12 wk
66/M	Lung carcinoma	Proven pulmonary aspergillosis	1	TR/L98H	4	None	VCZ	Died
59/M	Hematologic malignancy, allo-SCT, GvHD	Proven pulmonary aspergillosis	4	TR/L98H	8	VCZ (>1 mo)	VCZ	Died
54/M	Acute myeloid leukemia, relapse, allo-HSCT	Proven pulmonary aspergillosis	1	TR/L98H	8	ITZ (2–4 wk)	VCZ	Died
50/M	Non-Hodgkin lymphoma, allo-SCT, GvHD, lung cavities	Probable pulmonary aspergillosis	2	TR/L98H	16	VCZ (>1 mo)	VCZ	Died
36/F	Breast carcinoma with metastasis	Probable pulmonary aspergillosis	1	TR/L98H	1	None	VCZ	Died
13/F	Non-Hodgkin lymphoma	Proven pulmonary and CNS aspergillosis	1	TR/L98H	16	None	VCZ, CAS, AMB	Died
58/M	Liver transplantation for hepatic failure after methotrexate treatment for arteritis	Proven pulmonary and CNS aspergillosis	5	TR/L98H	2	None	AMB, VCZ	Died
60/M	Acute myeloid leukemia, allo-SCT, GvHD	Proven pulmonary and CNS aspergillosis	3	TR/L98H	4	FCZ (1–2 wk)	VCZ, CAS, AMB, POS	Survived

*VCZ, voriconazole; allo-SCT, allogeneic hematopoietic stem cell transplantation; GvHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; ITZ, itraconazole; CNS, central nervous system; CAS, caspofungin; AMB, amphotericin B; FCZ, fluconazole; POS, posaconazole.
† All cultures were *Aspergillus fumigatus*.
‡Azole treatment <12 wk before the first culturing of an azole-resistant isolate.
§Azole treatment after first culturing of resistant isolate.

showing azole resistance in 10 (9.5%) of 105 patients with a positive *Aspergillus* spp. culture. In a previous study, we reported a resistance prevalence of 12.8% among *A. fumigatus* isolates sent to our department by other hospitals in the Netherlands (*6*). This higher resistance rate may have been due to a different selection of isolates. In this study, all *Aspergillus* spp. isolates cultured in the participating laboratories were screened for resistance, irrespective of their clinical relevance, while in our previous study the isolates that were sent to Nijmegen were from patients with *Aspergillus* spp. diseases for whom antifungal therapy was not successful (*6*).

Two other studies have investigated the prevalence of azole resistance in *Aspergillus* spp. in the Netherlands. One study investigated a collection of 170 clinical *A. fumigatus* isolates recovered from 114 patients from 21 different medical centers in 1945–1998 (*30*). Three (1.8%) isolates were resistant to ITZ and were later found to harbor the TR/L98H resistance mechanism. These were the first TR/L98H isolates recovered in the Netherlands (*6*). Another study investigated the prevalence of azole-resistant *Aspergillus* spp. isolates in the Netherlands by using a collection of 209 unselected clinical *A. fumigatus* isolates obtained over a 6-month period in 2005 (*31*). Only 4 azole-resistant isolates were found, which corresponds with a prevalence of 1.9% (*31*). The low prevalence may be because of the methods used. We used ITZ-containing agar-slants to select for resistant isolates, which enables detection of resistant colonies even in the presence of multiple azole-susceptible colonies. Although Klaassen et al. (*31*) did not explain how isolates were obtained, azole-resistant colonies could have been missed if regular culture media were used (*22*,*31*). A κ coefficient of 0.81 indicates that screening for azole resistance by using agar supplemented with ITZ is a highly reproducible method and appears to be a feasible approach for screening for azole resistance and for surveillance studies (*34*).

Most (74/82 [90.2%]) of the azole-resistant isolates in our study were found to possess the TR/L98H mutation in the *cyp51A*-gene, which is a similar proportion as reported previously (*6*). Previous studies have suggested that the probability of 2 genomic changes developing during azole therapy is highly unlikely and that exposure of *A. fumigatus* to azoles in the environment may be a possible route of resistance development (*6*,*27*,*28*). *A. fumigatus* isolates harboring the TR/L98H resistance mechanism were recovered from the environment and were genetically related to TR/L98H-containing clinical isolates (*28*). This mode of resistance development implies that previous exposure of patients to azole compounds is not a prerequisite. Indeed, our study shows that 64% of patients with an azole-resistant isolate have no history of previous azole exposure during the 3 months before culturing the isolate. This finding supports the proposed environmental route of resistance development.

The prevalence and spread of TR/L98H in *A. fumigatus* in other countries are largely unknown. A recent study by Mortensen et al. showed that 8% of environmental *A.*

fumigatus isolates in Denmark harbored the TR/L98H resistance mechanism, which indicates that TR/L98H may be spreading (*29*). Because in vitro susceptibility testing of *A. fumigatus* is not routinely performed in most laboratories, international surveillance studies are warranted.

Most TR/L98H isolates were resistant to voriconazole (79%), but a broad range of MICs was observed that varied from 1 mg/L to 16 mg/L. Reasons for this variation remain unclear. TR/L98H isolates may have developed additional mutations that confer voriconazole resistance but that are not associated with the *cyp51A* gene. Another possibility is that the tandem repeat, which serves as a transcriptional enhancer, causes varying levels of up-regulation of the *cyp51A* gene. This up-regulation may result in differences in the voriconazole phenotype. More research is needed to gain insight into this phenomenon.

The outcome for patients with azole-resistant invasive aspergillosis was dismal, with a mortality rate of 88%. None of the patients who were treated with voriconazole monotherapy were alive at 3 months; of 3 patients whose treatment was switched to another class of antifungal drugs, only 1 survived. A critical issue for future study is whether azole resistance is a determinant of the poor clinical outcome of patients with azole-resistant aspergillosis. A previously reported case series of patients with primarily noninvasive *Aspergillus* spp. disease indicated that azole resistance was associated with treatment failure (*7*). However, establishing a relation between resistance and treatment outcome in invasive aspergillosis is complex because of the multiple factors that contribute to treatment outcome, including underlying disease, drug exposure, timing and accuracy of diagnosis, and timing of antifungal therapy. Furthermore, data are lacking on the effects of in vitro susceptibility patterns of *Aspergillus* spp. isolates on treatment outcomes. This relationship was recently described by using *Aspergillus* spp. isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network (*35*). The 12-week mortality rate for patients with culture-positive confirmed or probable invasive aspergillosis was 53% (*35*). For patients with azole-susceptible *A. fumigatus* aspergillosis treated with voriconazole, the proportion of deaths was 48% (J.W. Baddley, pers. comm.).

In a study by Herbrecht et al. (*2*), a subset of 51 modified-intent-to-treat patients with definite (43 patients) or probable (8 patients) invasive aspergillosis had a positive culture with *A. fumigatus* with a wild-type susceptibility. The all-cause proportion of deaths in this group at 12 weeks was 39% (P. Troke, pers. comm.). Both these analyses show a lower death rate than observed in our study, which indicates that azole resistance may be associated with a poor outcome. The median MIC of voriconazole in the 8 patients with azole-resistant invasive aspergillosis was 4 mg/L, which is resistant (*20*). Effects of an elevated voriconazole MIC on treatment outcome were supported by preclinical experimental results, in which mice infected with an *A. fumigatus* isolate that had a voriconazole MIC of 2 mg/L required a 2-fold higher voriconazole dose than did mice infected with a wild-type isolate to achieve a comparable survival rate (*25*).

The low survival rate of patients whose treatment was switched from azole therapy to nonazole therapy could be due to the delay of initiation of treatment with an effective alternative drug or to the presence of cerebral disease. Overall, the number of patients with azole-resistant aspergillosis in our study was low, and additional studies are needed to confirm the relation between azole resistance and treatment failure.

Because specimens from patients with invasive aspergillosis seldom produce positive cultures, our study underestimates the number of azole-resistant cases. The diagnosis of azole resistance in culture-negative cases is a challenge because current biomarkers, such as galactomannan and 1,3-β-D-glucan, do not enable species identification, let alone in vitro susceptibility testing. Molecular tools have been shown to be able to detect mutations associated with azole resistance directly in tissue (*36*) or in respiratory specimens (*37*) but are not yet suitable for use in routine patient care. Molecular tools need to be developed that enable the rapid detection of multiple mutations, although only known mutations can be detected.

Our study shows that azole resistance in clinical *A. fumigatus* isolates is widespread in the Netherlands and that the survival rate of patients with azole-resistant invasive aspergillosis is low. The dominance of the TR/L98H resistance mechanism and the high proportion of resistant isolates recovered from azole-naive patients support an environmental route of resistance development. We believe that continued surveillance is required in the Netherlands, as well as routine in vitro susceptibility testing of *A. fumigatus* isolates obtained from patients with *Aspergillus* spp. disease. International surveillance programs are also warranted to estimate the size of the emerging problem of azole resistance. Furthermore, the first-line therapy of patients with invasive aspergillosis should be reconsidered, at least in those centers with a high prevalence of azole resistance.

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Invasive Non-*Aspergillus* Mold Infections in Transplant Recipients, United States, 2001–2006

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Learning Objectives

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

- Distinguish the type of fungal infection most common in the current case series
- Evaluate patient characteristics associated with fungal infection after transplant
- Assess other factors associated with fungal infection after transplant
- Analyze the epidemiology of mucormycosis in the current case series

Editor

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Recent reports describe increasing incidence of non-*Aspergillus* mold infections in hematopoietic cell transplant (HCT) and solid organ transplant (SOT) recipients. To investigate the epidemiology of infections with Mucorales, *Fusarium* spp., and *Scedosporium* spp. molds, we analyzed data from the Transplant-Associated Infection Surveillance Network, 23 transplant centers that conducted prospective surveillance for invasive fungal infections during 2001–2006. We identified 169 infections (105 Mucorales, 37 *Fusarium* spp., and 27 *Scedosporium* spp.) in 169 patients; 124 (73.4%) were in HCT recipients, and 45 (26.6%) were in SOT recipients. The crude 90-day mortality rate was 56.6%. The 12-month mucormycosis cumulative incidence was 0.29% for HCT and 0.07% for SOT. Mucormycosis incidence among HCT recipients varied widely, from 0.08% to 0.69%, with higher incidence in cohorts receiving transplants during 2003 and 2004. Non-*Aspergillus* mold infections continue to be associated with high mortality rates. The incidence of mucormycosis in HCT recipients increased substantially during the surveillance period.

Invasive mold infections are a major source of illness and death in transplant recipients. Non-*Aspergillus* invasive infections are of particular concern because of the following factors: the difficulty in distinguishing them clinically from *Aspergillus* spp. infections and from each other; their progressive and aggressive course; and the intrinsic resistance of many of these fungi to several antimicrobial agents, including voriconazole. Recent reports have described an increase at some medical centers in non-*Aspergillus* mold infections, particularly mucormycosis (formerly zygomycosis), often in persons receiving antifungal agents that have activity against *Aspergillus* spp. (*1*–*4*).

Most reports have come from single institutions and might not be broadly representative of general trends. A comprehensive look at the modern epidemiology of these infections, including trends at multiple sites, can increase understanding of their public health implications. The Transplant-Associated Infection Surveillance Network (TRANSNET), a multicenter network of 23 academic tertiary care medical centers in the United States, performed prospective surveillance for invasive fungal infections (IFIs) during 2001–2006 (*5*–*7*). We analyzed the uniquely comprehensive TRANSNET cohort for epidemiology, patient demographics, clinical features, and outcomes of infections caused by the most common non-*Aspergillus* invasive molds detected: those of the order Mucorales, *Fusarium* spp., and *Scedosporium* spp.

Methods

Study personnel based at each TRANSNET center reviewed records of patients who had undergone solid organ transplantation (SOT) or hematopoietic cell transplantation (HCT). Each IFI was evaluated to determine whether it met the criteria for a proven or probable IFI as defined by the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC-MSG) (*8*). Trained personnel at each site completed a detailed case report form for each identified proven and probable IFI. Data included demographics, clinical characteristics, clinical involvement, underlying diseases and conditions, and use of antifungal drugs. Investigators collected clinical data on all HCT recipients in whom an IFI developed, regardless of transplant date. Surveillance began in March 2001 and concluded in March 2006.

Cultures and histopathologic specimens were processed at the participating hospitals. Species were identified by using routine methods at the local laboratories. Fungal isolates were forwarded to the University of Alabama at Birmingham Fungal Reference Laboratory (Birmingham, AL, USA) and the Mycotic Diseases Branch at the Centers for Disease Control and Prevention (Atlanta, GA, USA), where species identification was confirmed by using morphologic and DNA-based methods. Patients in this analysis had an IFI and 1) a culture or histopathologic specimen consistent with Mucorales mold or 2) a culture from a clinical specimen positive for *Fusarium* spp. or *Scedosporium* spp. We excluded patients with invasive mold infection for whom diagnostic evidence was inconclusive.

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All detected cases, regardless of transplant date, were described (surveillance cohort). In addition, the subset of cases in persons receiving transplants during the surveillance period was included for determination of the incidence of these infections (incidence cohort). Denominator data collected on identified patients undergoing transplant at each site during the surveillance period included date and type of transplant and date of last follow-up and clinical status at that time (alive, had undergone retransplant, IFI, dead). These data were used to calculate transplant-associated 12-month cumulative incidence. Estimated cumulative incidence was based on time to first infection after transplant and accounted for the competing risks for death, relapse of underlying disease, and retransplant. For HCT recipients, overall and transplant-specific 12-month cumulative incidence was calculated for mucormycosis and for fusariosis and scedosporiosis combined. For SOT patients, overall and transplant-specific incidence was estimated only for mucormycosis because of low numbers of scedosporiosis and fusariosis. We estimated cumulative incidence using the cmprisk risk package version 2.2-1 in R version 2.11.1 (www.r-project.org).

We also explored the change in incidence of mold infections over time among HCT recipients. Because case counts can vary due to increases or decreases in the size of the denominator, we measured the changing epidemiology by estimating the incidence, according to transplant date. To do this, we classified the denominator of all transplant patients into 9 sequential subcohorts according to the 4-month period when the transplant was performed, as described (*5*,*7*). Twelve-month cumulative incidence for each of the 9 time periods (i.e., subcohorts) was calculated for mucormycosis and for fusariosis and scedosporiosis combined.

In addition, we attempted to estimate whether the underlying risk for infection in the overall population was changing because of changes in the numbers of allogeneic HCTs. To do this, we calculated the total follow-up time (in person-days) for each transplant type and plotted it for each period. Analysis was limited to the 21 sites that contributed data consistently during May 2002–April 2005, the last period for which complete data were available.

Results

Clinical Description of Cases

Of the 1,208 cases of proven or probable IFI in SOT recipients (*7*) and the 983 cases in HCT recipients (*5*) in TRANSNET, we detected 169 cases of Mucorales, *Fusarium* spp., or *Scedosporium* spp. infection in 166 transplant recipients, making these molds the most frequently identified molds, after *Aspergillus* within this patient population. The Mucorales (105 patients) were the most common of these molds, followed by *Fusarium* spp. (37 patients), and *Scedosporium* spp. (27 patients). Most cases occurred among HCT recipients (124 [73.4%] patients), compared with 45 (26.6%) cases in SOT recipients (Table 1).

Among the Mucorales, *Rhizopus* was the most common genus (55 [52.4%] of 105 cases), followed by *Mucor* (19 [18.1%]) and unspecified Mucorales (10 [9.5%]) (Table 1). *F. solani* (10 [27.0%] cases) was the most common *Fusarium* species; however, we detected a large number of unspecified *Fusarium* spp. (22 [59.5%]). *S. apiospermum* (19 [70.4%]) and *S. prolificans* (8 [29.6%]) were the most common *Scedosporium* species (27 cases).

Site Distribution

Numbers of cases varied by hospital and by region (Table 2). The 3 northeastern hospitals collectively reported 12 (7.1%) cases of non-*Aspergillus* mold infections during the surveillance period; 7 were caused by Mucorales. The 11 hospitals in the South reported 80 (47.3%) cases, of which 46 were caused by Mucorales. In the Midwest, the 5 participating hospitals detected 51 (30.2%) cases, and the 3 western hospitals detected 26 (15.4%) cases. The total number of Mucorales, *Fusarium* spp., or *Scedosporium* spp. infections varied substantially by site, ranging from 0 cases (3 sites) to 31 cases (1 site [18.3% of total cases]). The total number of transplants at each site for which follow-up was conducted as part of TRANSNET also varied widely,

Table 1. Distribution of Mucorales, *Fusarium*, and *Scedosporium* organisms causing infection in HCT and SOT recipients, as detected in TRANSNET, United States, 2001–2006*

Organism	No. (%) patients		
	HCT	SOT	Total
Mucorales	77 (62.1)	28 (62.2)	105 (62.1)
Rhizopus spp.	39 (50.6)	16 (57.1)	55 (52.4)
Mucor spp.	12 (15.6)	7 (25.0)	19 (18.1)
Rhizomucor spp.	7 (9.1)	0	7 (6.7)
Cunninghamella spp.	5 (6.5)	4 (14.3)	9 (8.6)
Lichtheimia spp.†	3 (3.9)	0	3 (2.9)
Apophysomyces spp.	1 (1.3)	0	1 (1.0)
Syncephalastrum spp.	1 (1.3)	0	1 (1.0)
Unspecified	9 (11.7)	1 (3.6)	10 (9.5)
Genus *Fusarium*	31 (25.0)	6 (13.3)	37 (21.9)
F. solani	9 (29.0)	1 (16.7)	10 (27.0)
F. oxysporum	0	2 (33.3)	2 (5.4)
F. proliferatum	2 (6.5)	0	2 (5.4)
F. verticilloides	1 (3.2)	0	1 (2.7)
Unspecified	19 (61.3)	3 (50.0)	22 (59.5)
Genus *Scedosporium*	16 (12.9)	11 (24.4)	27 (16.0)
S. apiospermum	13 (81.3)	6 (54.5)	19 (70.4)
S. prolificans	3 (18.8)	5 (45.5)	8 (29.6)
Total	124 (73.4)	45 (26.6)	169 (100)

*HCT, hematopoietic cell transplant; SOT, solid organ transplant; TRANSNET, Transplant-Associated Infection Surveillance Network.
†This genus was formerly *Absidia.*

ranging from 89 to 2,551 HCTs and from 239 to 2,111 SOTs (Table 2). The median number of cases at a site was 8 (interquartile range 2–11 cases).

Patient Characteristics (Surveillance Cohort)

Of the 169 case-patients, 96 (56.8%) were male, and 141 (91.6%) were white (Table 3); median age was 49 years (range 36–57 years). Proven IFIs comprised 95 (56.2%) cases. Active diabetes at the time of infection, either glucocorticoid-associated or as a preexisting condition, was present among 46 (43.8%) mucormycosis patients, 9 (56.8%) fusariosis patients, and 7 (25.9%) scedosporiosis patients. The overall crude mortality rate at 90 days was 56.6% (90/169).

The lower respiratory tract was the most commonly involved site for all mold illnesses (53.1% of mucormycosis, 38.9% of fusariosis, and 59.3% of scedosporiosis) (Table 3). Disseminated disease (17.4% of all cases) was also frequent (13.3% of mucormycosis, 22.2% of fusariosis, and 25.9% of scedosporiosis).

Of the 124 HCT recipients with 1 of these infections, the most common indication for HCT was acute myelogenous leukemia (35 [28.2%] cases), followed by non-Hodgkin lymphoma (26 [21.0%] cases) (Table 3). Most HCT recipients received a human leukocyte antigen (HLA)–matched, allogeneic graft from a related donor (54 [43.5%] patients), followed by HCT recipients with an unrelated donor (47 [37.9%] patients) (Table 3). In the 60 days before diagnosis of infection, neutropenia was reported in 4 (25.0%) patients with scedosporiosis, 39 (50.6%) with mucormycosis, and 17 (54.8%) with fusariosis. Acute graft-versus-host disease was present in 32 (41.6%) patients with mucormycosis, 7 (22.6%) with fusariosis, and 6 (37.5%) with scedosporiosis.

Of the 45 transplant patients with mold infections who had received an SOT, 19 (42.2%) had received a lung and 12 (26.7%) had received a kidney (Table 3). Twenty-four (54.5%) patients experienced organ rejection in the 30 days before infection occurred.

Many cases occurred early after transplant, but late-onset infection was also prevalent. Among HCT recipients, 67 (54.4%) infections occurred <6 months after transplant; 17 (13.8%) occurred ≥2 years after transplant (Figure 1). Among SOT recipients, 17 (37.8%) infections occurred within the first 6 months; 15 (33.3%) occurred ≥2 years after transplant. Median time to IFI for liver transplant

Table 2. Regional and hospital variability of Mucorales, *Fusarium* spp., and *Scedosporium* spp. invasive infections, as reported in TRANSNET, United States, 2001–2006*

US region,† hospital code	No. transplant recipients		No. (%) cases			
	HCT	SOT	Mucorales, n = 105	*Fusarium* spp., n = 37	*Scedosporium* spp., n = 27	Total, N = 169
Northeast			7 (6.7)	3 (8.1)	2 (7.4)	12 (7.1)
A	608	1,532	5 (4.8)	1 (2.7)	1 (3.7)	7 (4.1)
B	245	377	1 (1.0)	2 (5.4)	1 (3.7)	4 (2.4)
C	1,107	NA	1 (1.0)	0	0	1 (0.6)
South			46 (43.8)	23 (62.2)	11 (40.7)	80 (47.3)
D	2,551	NA	18 (17.1)	12 (32.4)	1 (3.7)	31 (18.3)
E	646	549	6 (5.7)	3 (8.1)	6 (22.2)	15 (8.9)
F	363	NA	5 (4.8)	3 (8.1)	1 (3.7)	9 (5.3)
G	523	1,201	4 (3.8)	2 (5.4)	2 (7.4)	8 (4.7)
H	342	1,532	5 (4.8)	1 (2.7)	1 (3.7)	7 (4.1)
I	808	377	4 (3.8)	0	0	4 (2.4)
J	89	728	3 (2.9)	0	0	3 (1.8)
K	2,040	239	1 (1.0)	1 (2.7)	0	2 (1.2)
L	110	1,210	0	1 (2.7)	0	1 (0.6)
M	449	NA	0	0	0	0
N	511	NA	0	0	0	0
Midwest			35 (33.3)	9 (24.3)	7 (25.9)	51 (30.2)
O	970	2,111	8 (7.6)	5 (13.5)	4 (14.8)	17 (10.1)
P	1,028	1,391	8 (7.6)	2 (5.4)	0	10 (5.9)
Q	361	1,210	8 (7.6)	0	0	8 (4.7)
R	315	2,111	5 (4.8)	0	3 (11.1)	8 (4.7)
S	546	755	6 (5.7)	2 (5.4)	0	8 (4.7)
West			17 (16.2)	2 (5.4)	7 (25.9)	26 (15.4)
T	1,512	NA	9 (8.6)	2 (5.4)	4 (14.8)	15 (8.9)
U	854	NA	8 (7.6)	0	3 (11.1)	11 (6.5)
V	1,019	NA	0	0	0	0

*TRANSNET, Transplant-Associated Infection Surveillance Network; HCT, hematopoietic cell transplant; SOT, solid organ transplant; NA, not available because of nonparticipation in surveillance for this transplant type.
†Northeast: Massachusetts, New York, Pennsylvania; South: Alabama, Arkansas, Florida, Georgia, Maryland, North Carolina, Tennessee, Texas; Midwest: Iowa, Michigan, Minnesota, Nebraska, Wisconsin; West: California, Washington.

Table 3. Characteristics of HCT and SOT patients* with proven or probable invasive Mucorales, *Fusarium* spp., or *Scedosporium* spp. infection, as reported in TRANSNET, United States, 2001–2006†

Characteristic‡	Mucorales, no. (%), n = 105	*Fusarium* spp., no. (%), n = 37	*Scedosporium* spp., no. (%), n = 27	Total, no. (%), N = 169
Male sex	58 (56.9)	26 (70.3)	12 (44.4)	96 (57.8)
White race	86 (90.5)	34 (94.4)	21 (91.3)	141 (91.6)
Proven IFI	61 (58.1)	21 (56.8)	13 (48.1)	95 (56.2)
Diabetes mellitus at time of fungal infection	46 (43.8)	9 (56.8)	7 (25.9)	62 (36.7)
Prior IFI	23 (21.9)	8 (19.5)	10 (24.4)	41 (24.3)
Prior aspergillosis	17 (16.2)	1 (2.7)	4 (14.8)	22 (13.0)
Antemortem clinical involvement§				
Pulmonary only	52 (53.1)	14 (38.9)	16 (59.3)	82 (50.9)
Sinus only	12 (12.2)	2 (5.6)	1 (3.7)	15 (9.3)
Skin only	9 (9.2)	3 (8.3)	0	12 (7.5)
Disseminated	13 (13.3)	8 (22.2)	7 (25.9)	28 (17.4)
Rhinocerebral only	3 (3.1)	1 (2.8)	0	4 (2.5)
Other¶	3 (3.1)	6 (16.7)	2 (7.4)	11 (6.8)
Death at 90 d	57 (58.2)	18 (51.4)	15 (57.7)	90 (56.6)
HCT	77 (73.3)	31 (24.3)	16 (59.3)	124 (73.4)
Underlying condition				
ALL	7 (9.1)	1 (3.2)	2 (12.5)	10 (8.1)
AML	21 (27.3)	11 (35.5)	3 (18.8)	35 (28.2)
CML	7 (9.1)	2 (6.5)	4 (25)	13 (10.5)
HL	4 (5.2)	1 (3.2)	0	5 (4)
NHL	15 (19.5)	8 (25.8)	3 (18.8)	26 (21)
AA	3 (3.9)	1 (3.2)	0	4 (3.2)
MM	5 (6.5)	1 (3.2)	1 (6.3)	7 (5.6)
MDS	10 (13)	1 (3.2)	1 (6.3)	12 (9.7)
HCT graft donor				
Autologous	8 (10.4)	4 (12.9)	0	12 (9.7)
Allogeneic matched-related	34 (44.2)	13 (41.9)	7 (43.8)	54 (43.5)
Allogeneic mismatched-related	4 (5.2)	4 (12.9)	2 (12.5)	10 (8.1)
Allogeneic unrelated	30 (39)	10 (32.3)	7 (43.8)	47 (37.9)
Other characteristics				
Neutropenia#	39 (50.6)	17 (54.8)	4 (25)	60 (48.4)
Steroids at diagnosis	59 (76.6)	24 (77.4)	14 (87.5)	97 (78.2)
Myeloablative preconditioning	46 (59.7)	18 (58.1)	11 (68.8)	75 (60.5)
Acute GvHD	32 (41.6)	7 (22.6)	6 (37.5)	45 (36.3)
Chronic GvHD	29 (37.7)	14 (45.2)	7 (43.8)	50 (40.3)
Steroid treatment	58 (75.3)	19 (61.3)	13 (81.3)	90 (72.6)
GvHD grade 1 or 2	18 (23.4)	4 (12.9)	4 (25.0)	26 (21.0)
GvHD grade 3 or 4	35 (45.4)	14 (45.2)	5 (31.3)	54 (43.5)
SOT	28 (26.7)	6 (51.4)	11 (40.7)	45 (26.6)
Organ transplanted				
Heart	3 (10.7)	1 (16.7)	0	4 (8.9)
Lung	8 (28.6)	2 (33.3)	9 (81.8)	19 (42.2)
Kidney	9 (32.1)	1 (16.7)	2 (18.2)	12 (26.7)
Liver	9 (32.1)	2 (33.3)	0	11 (24.4)
Organ rejection within previous 30 d	13 (48.1)	3 (50)	8 (72.7)	24 (54.5)

*Includes 3 patients with 2 infections each; 2 patients had separate *Fusarium* and *Scedosporium* infections; 1 person had separate mucormycosis and scedosporiosis.
†Denominators vary because of missing data. HCT, hematopoietic cell transplant; SOT, solid organ transplant; TRANSNET, Transplant-Associated Infection Surveillance Network; IFI, invasive fungal infection; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; AA, aplastic anemia; MM, multiple myeloma; MDS, myelodysplastic syndrome; GvHD, graft-versus-host disease.
‡Median ages (interquartile ranges) are as follows: patients with Mucorales infection, 51 y (39–57 y); patients with *Fusarium* spp. infection, 45 y (32–59 y); patients with *Scedosporium* spp. infection, 46 y (38–59 y); and all patients, 49 y (36–57 y).
§Antemortem organ involvement was classified according to recorded sites of infection; disseminated infection was defined as infection in noncontiguous organs.
¶Esophageal (1 *Scedosporium* spp.); heart (3 Mucorales); blood (6 *Fusarium* spp., 1 *Scedosporium* sp.).
#Defined as an absolute neutrophil count <500/μL, within 60 d before development of invasive fungal infection.

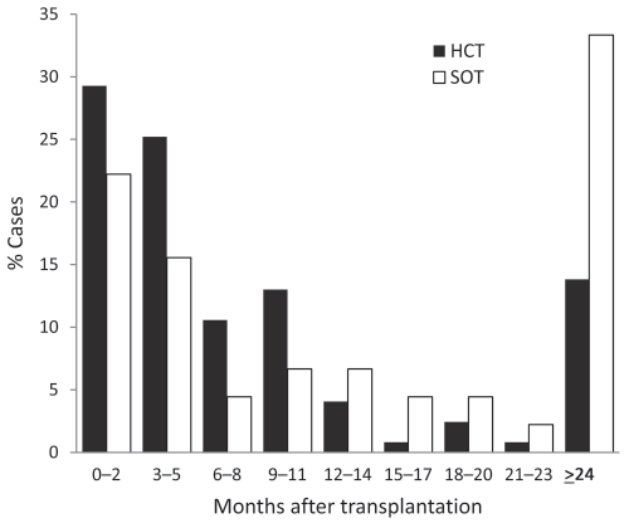

Figure 1. Months from transplant to development of invasive mucormycosis, fusariosis, or scedosporiosis among hematopoietic cell (HCT) and solid organ (SOT) transplant recipients as reported in the Transplant-Associated Infection Surveillance Network, United States, 2001–2006.

recipients was 81 days, compared with 533 for non–liver SOT recipients (p = 0.035).

Exposure to Antifungal Drugs

Voriconazole was the most frequently administered antifungal agent before infection in mucormycosis patients (47 [44.7%]) (Table 4). In contrast, fewer patients with fusariosis (27.0%) or scedosporiosis (11.1%) had received voriconazole (Table 4). Of the 47 patients with mucormycosis who received voriconazole, 28 (59.6%) were receiving voriconazole as prophylaxis, 15 (31.9%) were receiving it as empiric therapy, and 4 (8.7%) were receiving it as treatment for a suspected or other proven fungal infection.

More mucormycosis patients received an amphotericin B formulation after diagnosis (78.1%) than did fusariosis (51.4%) and scedosporiosis (33.3%) patients (Table 4). Patients with fusariosis (20 [54.1%] of 37) and scedosporiosis (15 [55.6%] of 27) most commonly received voriconazole after IFI diagnosis.

Incidence Cohort

Follow-up data were available for 15,820 HCT recipients from 22 sites. Among these, 17% died, 13% experienced relapse, and 1% underwent retransplant in the first 12 months after transplant. Mucormycosis was diagnosed in 44 (0.3%) persons. Overall 12-month cumulative incidence for mucormycosis in HCT recipients was 0.29% (Figure 2). Recipients of an organ or cells from an allogeneic HLA-unrelated donor had the highest 12-month cumulative incidence of mucormycosis at 0.85%; allogeneic HLA-matched related donors had a cumulative incidence of 0.58%; and allogeneic HLA-mismatched related donors had a 12-month cumulative incidence of 0.25% (Figure 2).

Follow-up data were available for 16,457 SOT recipients from 15 sites. Of these, 6% died; 2% of kidney transplant recipients returned to chronic dialysis; and 2% underwent retransplant within 12 months after the transplant. In 12 (0.07%) persons, mucormycosis was diagnosed within the first 12 months after transplant. In SOT recipients, the overall 12-month cumulative incidence of mucormycosis was 0.07% (Figure 3). Patients receiving lung transplants and liver transplants had the highest incidence of mucormycosis (0.18% and 0.16%,

Table 4. Antifungal medications received before and after diagnosis of invasive mold infection, TRANSNET data, United States, 2001–2006*

Antifungal drug	Mucorales, no. (%), n = 105	*Fusarium* spp., no. (%), n = 37	*Scedosporium* spp., no. (%), n = 27	Total, no. (%), N = 169
Received before diagnosis				
Amphotericin B†	7 (6.7)	5 (13.5)	3 (11.1)	15 (8.9)
Fluconazole	35 (33.3)	17 (45.9)	5 (18.5)	57 (33.7)
Itraconazole	13 (12.4)	4 (10.8)	8 (29.6)	25 (14.8)
Voriconazole	47 (44.8)	10 (27.0)	3 (11.1)	60 (35.5)
Caspofungin	18 (17.1)	3 (8.1)	3 (11.1)	24 (14.2)
Posaconazole	0	1 (2.7)	0	1 (0.6)
Received after diagnosis				
Amphotericin B†	82 (78.1)	19 (51.4)	9 (33.3)	110 (65.1)
Fluconazole	4 (3.8)	0	0	4 (2.4)
Itraconazole	10 (9.5)	2 (5.4)	8 (29.6)	20 (11.8)
Voriconazole	39 (37.1)	20 (54.1)	15 (55.6)	74 (43.8)
Caspofungin	26 (24.8)	12 (32.4)	11 (40.7)	49 (29.0)
Posaconazole	24 (22.9)	3 (8.1)	3 (11.1)	30 (17.8)

*TRANSNET, Transplant-Associated Infection Surveillance Network. Numbers do not total 100% because of concomitant receipt of multiple antifungal agents.
†Any amphotericin B formulation.

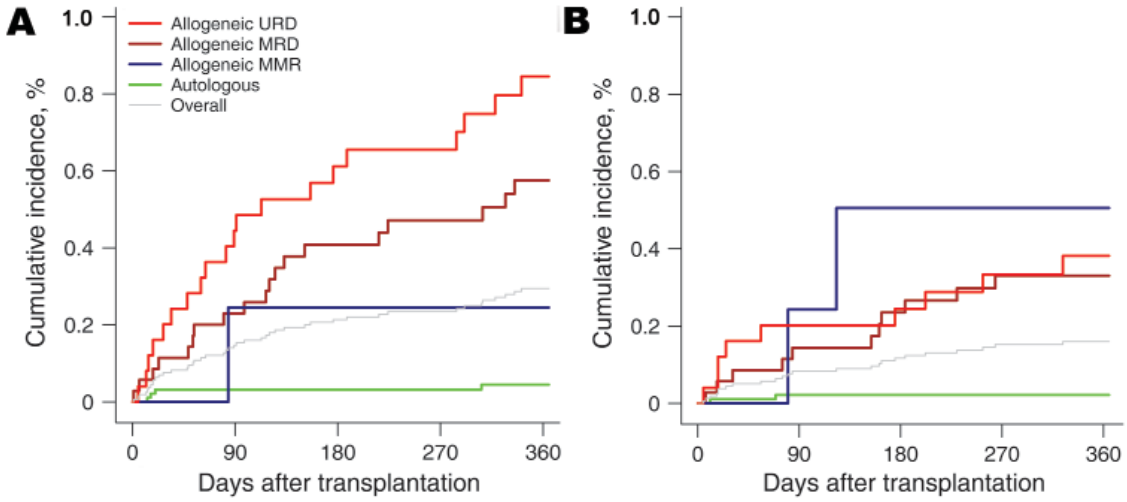

Figure 2. Twelve-month cumulative incidence for invasive A) Mucorales infections and B) combined *Fusarium* and *Scedosporium* spp. infections among donor types of hematopoietic cell transplant recipients reported in the Transplant-Associated Infection Surveillance Network, United States 2001–2006. Infection data by transplant type: allogenic URD, 2,483 patients, 20 with Mucorales infection, 9 with *Fusarium* or *Scedosporium* spp. infection; allogenic MRD, 3,499 patients, 19 with Mucorales infection, 11 with *Fusarium* or *Scedosporium* spp. infection; allogenic MMR, 427 patients, 1 with Mucorales infection, 2 with *Fusarium* or *Scedosporium* spp. infection; autologous, 9,326 patients, 4 with Mucorales infection, 2 with *Fusarium* or *Scedosporium* spp. infection; overall, 15,820 patients, 44 with Mucorales infection, 24 with *Fusarium* or *Scedosporium* spp. infection. URD, unrelated donor; MRD, matched related donor; MMR, mismatched related donor.

respectively). Four *Scedosporium* spp. (3 lung SOTs, 1 kidney) and 2 *Fusarium* spp. (1 lung SOT, 1 liver) infections occurred within the first 12 months after transplant (data not shown).

We estimated cumulative incidence for mucormycosis and for *Fusarium* spp. and *Scedosporium* spp. infections combined for the 9 subcohorts from May–August 2002 through January–April 2005 (Figure 4). Cohorts ranged from ≈1,200 to 1,400 transplant recipients each. Estimated cumulative incidence of *Fusarium* spp. and *Scedosporium* spp. infections in the 9 subcohorts did not increase over time. Incidence ranged from 0% during January–April 2004 and September–December 2004 to a high of 0.30% (4 infections) during May–August 2004.

The incidence of mucormycosis in HCT recipients varied, with generally higher incidences in 2003 and 2004 than in 2002. Initially, the 12-month cumulative incidence for mucormycosis was 0.08% (1 infection) for the cohort receiving transplants during May–August 2002. The cumulative incidence was higher for all subsequent cohorts and peaked at 0.69% (9 infections) in the cohort receiving transplants during September–December 2004. In the subsequent cohort that received transplants during January–April 2005, incidence declined to 0.16% (2 infections). These 39 Mucorales infections occurred at 13 of the 21 sites, with these 13 sites contributing 1–7 infections each. The pattern was similar across the 9 subcohorts when we excluded the site with most cases (site D) and limited the analysis to allogeneic transplants only (data not shown). In addition, the total follow-up time of allogeneic HCTs did not appreciably increase (Figure 4). Mucormycosis incidence rates appeared to decrease for the cohorts receiving transplants during the first 4 months (January–April) of each year in the 2003–2005 surveillance period.

Discussion

We describe the modern epidemiology of common non-*Aspergillus* invasive mold infections at multiple tertiary care sites throughout the United States. During the TRANSNET surveillance period, the estimated cumulative incidence of mucormycosis among HCT subcohorts receiving transplants in 2003 and 2004 was generally higher than in 2002. Public health officials and clinicians should be aware of these emerging mold infections, particularly in susceptible hosts, such as transplant recipients.

The worrisome possibility of an increase in mucormycosis incidence has gained attention recently, particularly because this disease has an extremely high case-fatality rate (*9*,*10*). Most supporting information about the emergence of this mold has come from compilations of case series (*11*,*12*), single-center studies (*13*–*15*), or registries (*16*,*17*). One national study in France used administrative data to demonstrate an increase over a 10-year period (*18*). In contrast, our analysis is based on a comprehensive database from a surveillance program that included a large number of centers with a broad geographic distribution. Furthermore, all cases were independently evaluated by using standardized case definitions (EORTC-MSG criteria [*8*]).

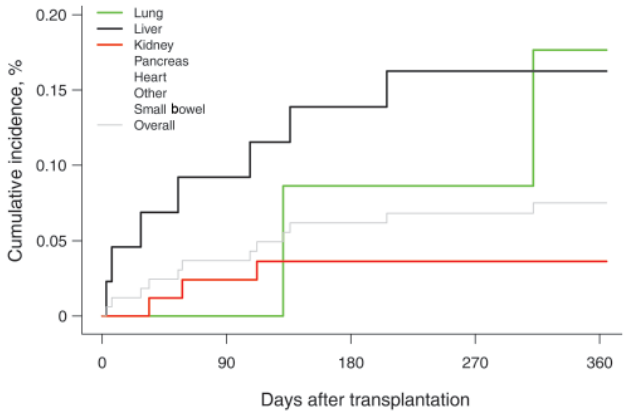

Figure 3. Twelve-month cumulative incidence for invasive Mucorales infections among solid organ transplant recipients reported in the Transplant-Associated Infection Surveillance Network, United States, 2001–2006. A total of 16,457 transplant patients had 12 Mucorales infections. Infection data by transplant type: lung, 1,179 patients, 2 with Mucorales infection; liver, 4,361 patients, 7 with Mucorales infection; kidney, 8,494 patients, 3 with Mucorales infection; pancreas, 1,174 patients, 0 with Mucorales infection; heart, 1,159 patients, 0 with Mucorales infection; other, 21 patients, 0 with Mucorales infection; small bowel, 69 patients, 0 with Mucorales infection.

Reasons for higher incidence of mucormycosis during 2003 and 2004 are not clear from these data. It is certainly possible that these are natural variations or that changes in practices (e.g., mold-active prophylaxis or immunosuppressive agents) or changes in the at-risk populations could be contributory factors. Although we did not see an increase in the numbers of transplants from allogeneic donors performed at the participating sites during this period, we did not have data on other conditions that increase risk for mucormycosis, such as diabetes mellitus or iron overload conditions. The higher incidence during certain periods did not result from a single institution; nor was it likely to be attributable to surveillance artifact, in which improved detection methods falsely suggest a changing trend because other common non-*Aspergillus* mold infections did not also increase during this period.

Another issue that has been reported recently is the frequency of breakthrough mucormycosis in patients receiving voriconazole (*3*,*4*,*19*–*21*). In these reports, progressive mucormycosis developed in patients who were receiving voriconazole for prophylaxis or treatment for another disease, or these patients were at higher risk for mucormycosis than were control populations (*2*). In our study, voriconazole was the most frequently reported antifungal drug used before mucormycosis developed, although it was used in fewer than half of all patients. The frequency of voriconazole use before development of mucormycosis is striking, especially in the context of these case reports; however, the implication of the broader use of this antifungal drug to the emergence of this mold is far from clear. Experimental models have demonstrated increased virulence of Mucorales after exposure to voriconazole (*22*), but other reports have noted an increase of this mold before introduction of voriconazole (*14*,*23*). In a randomized trial that used voriconazole as a prophylactic agent, incidence of mucormycosis was not higher among the intervention group than in the group who received fluconazole (*24*), although patients meeting enrollment criteria may not have been at high risk for mucormycosis (*25*). The increase might not be evidence of a causal relationship between voriconazole and mucormycosis but might instead reflect a parallel increase related to a changing patient risk profile, including patients at higher risk or increased time at risk, mainly through improved posttransplant survival (*25*). We did find that 1 factor associated with death in mucormycosis patients on bivariate analysis was prior use of voriconazole (data not shown); this finding could indicate that persons receiving voriconazole are more complicated transplant patients and therefore at higher underlying baseline risk for mucormycosis. Because our study did not collect medication data on uninfected controls or on global antimicrobial drug use practices, we were not able to correlate broader voriconazole use to the higher incidence of mucormycosis in this cohort.

We found a high degree of site-to-site variability in the number of cases reported. One hospital contributed 31 cases, 18 of which were caused by Mucorales; this number was

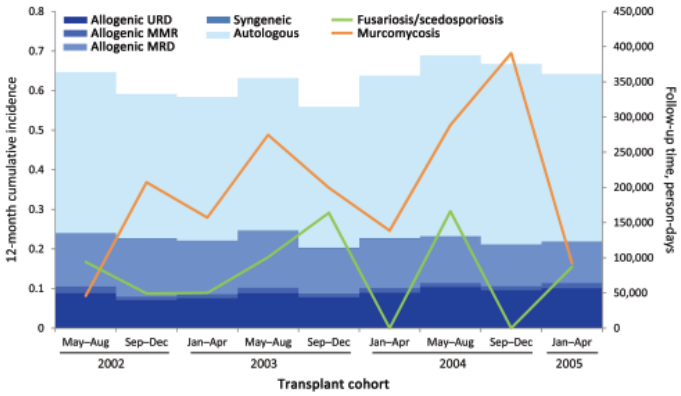

Figure 4. Changes in 12-month cumulative incidence for invasive Mucorales infections, compared with *Fusarium* and *Scedosporium* infections, reported in the Transplant-Associated Infection Surveillance Network, United States 2001–2005. Changes in the underlying hematopoietic cell transplant population, by transplant type, is shown for comparison. URD, unrelated donor; MMR, mismatched related donor; MRD, matched related donor.

nearly double that of the hospital with the second-highest number. No cases were detected at 3 sites. However, the site with 31 cases was also 1 of the sites with the highest number of HCTs performed per year. Whether this variability is a function of environmental or climate conditions, the underlying transplant population at that site, medical practices (e.g., prophylaxis, diagnostic interventions), or a combination of these factors is not known.

The variability in the incidence of infections from Mucorales in HCT recipients over time was also intriguing, with the lowest incidence in the cohorts who received transplants during the first 4 months of the year. One recent report described a similar finding for aspergillosis among HCT recipients (*26*). Whether this pattern reflects an underlying seasonal risk for mucormycosis is not known. However, a seasonal pattern is feasible; these molds exist in the environment, and environmental and temperature conditions can greatly influence their growth or sporulation (*27*). Further study of the seasonal risk for rare mold infections such as these most likely necessitates a larger cohort than that from TRANSNET; large national databases, including those using administrative codes, despite their poor predictive values (*28*), might be promising for estimating trends for such diseases.

Our data corroborate other reports regarding the clinical features of these mold infections (*11*,*29*–*33*). We found that even though most cases occurred soon after transplant, a large number occurred >6 months after transplant. A similar pattern occurs in other mold infections, such as those caused by *Aspergillus* spp., indicating the need for clinicians to be vigilant about mold infections, particularly a substantial length of time after transplant. Furthermore, we found that the clinical features of these 3 common non-*Aspergillus* mold infections were similar (with only bloodstream involvement being associated more often with fusariosis, as would be expected) (*30*). Not surprisingly, the pulmonary system and sinuses were the most common sites of infection for all molds studied, similar to infections caused by *Aspergillus* spp. These data highlight the similarities in features among mold infections and illustrate the difficulties in differentiating these infections on the basis of clinical features or organ involvement. Because correct treatment depends on the proper isolate identification, these results reinforce the need for major improvements in diagnostic testing, including development of tissue-based molecular identification methods (*34*,*35*) to correctly and efficiently identify the causative organism.

Our study had a few limitations. Because we collected data only on proven and probable IFIs (and omitted possible infections), as defined by EORTC-MSG criteria (*8*), we are likely to have underestimated disease in these populations. In addition, we were not able to systematically determine which, if any, of these infections resulted from donor-derived infection of the allograft. However, routine posttransplant surveillance conducted by transplant clinical teams, in conjunction with the United Network for Organ Sharing, did not indicate any such clusters.

Our large prospective parallel assessment of the modern epidemiology of these emerging, yet uncommon, non-*Aspergillus* molds demonstrated a generally higher incidence in mucormycosis in 2003 and 2004 than in 2002 in HCT recipients. More comprehensive and long-term surveillance for these emerging rare molds might be warranted to track trends and assess the changing landscape of these infections.

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Rickettsia honei Infection in Human, Nepal, 2009

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We report a case of *Rickettsia honei* infection in a human in Nepal. The patient had severe illness and many clinical features typical of Flinders Island spotted fever. Diagnosis was confirmed by indirect immunofluorescent assay with serum and molecular biological techniques. Flinders Island spotted fever may be an endemic rickettsiosis in Nepal.

Tick-borne rickettsioses are emerging zoonoses of marked endemicity caused by spotted fever group (SFG) rickettsia. Interest in rickettsioses is associated with increased description of new species and diseases, but this increase is unevenly distributed worldwide. Among patients with fever in Nepal, murine typhus and scrub typhus are frequently described (*1*), but tick-borne rickettsioses remain underinvestigated. *Rickettsia honei* is an SFG species that was described as a new species in 1998 and as the cause of Flinders Island spotted fever (FISF) in Australia (*2*,*3*). One human case of FISF has been confirmed in Thailand (*4*). We report a case of tick-borne rickettsiosis in Nepal caused by *R. honei* and highlight the necessity for heightened interest in emerging rickettsioses in Asia.

The Patient

A 67-year-old woman was admitted to the Canadian International Water and Energy Consultants Clinic Travel Medicine Center in Kathmandu, Nepal, in April 2009. She had a 5-day history of fever (40.3°C), headache, diarrhea, and severe arthralgias. Results of a physical examination were unremarkable. Laboratory tests showed a leukocyte count of 6,500 cells/mm^3, an increase in immmature neutrophils and polymorphonuclear leukocytes, and thrombocytopenia. Treatment was initiated with intravenous ceftriaxone, 2 g every 24 h for 8 days, for suspected enteric fever.

Within 48 hours, her condition worsened. The patient had photosensitivity, tinnitus, frontal headache, insomnia, confusion, cough, distress, hypotension, tachycardia, hypoxia (88% oxygenation with 2 L of O_2), and fever (38.4°C). She was also disoriented regarding place and time and had bilateral deafness, conjunctivitis, multiple lymphadenopathies, tender hepatosplenomegaly, bilateral rales, and a purpuric rash. The rash showed a predilection for the extremities, including palms and soles (Figure). There was no eschar. Pertinent laboratory values were the following: creatinine 2 mg/dL (baseline 0.8 mg/dL), aspartate aminotransferase 105 U/L, alkaline phosphatase 765 U/L, and minimum platelet count 40,000/mm^3. Chest radiograph showed bilateral interstitial infiltrates.

The patient was from New Zealand, had lived in Nepal for 30 years, and worked in wild dog protection. She reported removal of a tick 2 weeks before admission and contact with dogs, rats, ticks, fleas, and mosquitoes. She had returned from a 1-month visit to Queenstown, New Zealand, 3 months earlier and had stayed for 2 days in Thailand. She spent 1 year in Canberra, Australian Capital Territory, Australia, 12 years earlier. She was treated with oral doxycycline (100 mg 2×/d for 14 days) and showed defervescence by day 16. She recovered slowly over 3 months but had persistent tinnitus and residual high-tone hearing loss bilaterally.

Serum samples were sent to the Unité des Rickettsies (Marseille, France) to identify the etiologic agent. Samples were tested by using a multiple-antigen immunofluorescent test (*5*). Antigens included those from SFG *Rickettsia* spp., typhus group *Rickettsia* spp., and *Orientia tsutsugamushi*. Increased immunoglobulin (Ig) G and IgM titers were observed, mainly for SFG rickettsiae (Table). Kinetics of antibodies titers showed seroconversion within 3 weeks of follow-up and 4-fold increases in IgG titers, which confirm a diagnosis of rickettsial infection. As usually observed for *Rickettsia* species, serologic cross-reactivity occurred, but the highest increased antibodies titers in convalescent-phase serum were for *R. honei* (IgG 1,024, IgM 64) and *R. felis* (IgG 256, IgM 16) (Table).

The first serum sample negative for *Rickettsia* spp. was tested by real-time PCR. DNA was extracted from serum by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The result of a PCR using a probe specific for SFG *Rickettsia* spp. (*6*) was positive (cycle threshold 34.3). Rickettsial DNA was detected by PCR amplifications of the outer membrane protein A (*ompA*) and *ompB* genes of *Rickettsia* spp. We obtained amplification products of 514 bp and 100% similarity with the *R. honei ompA* gene (GenBank accession no. AF018075) and 603 bp with 100% similarity with the *R. honei ompB* gene (GenBank accession nos. AF123724 and AF123711) (*2*). We obtained 2 PCR products for 2 rickettsial genes, which showed 100% sequence similarity with *R. honei*. In addition, we detected increased antibodies titers for *R. honei* antigen

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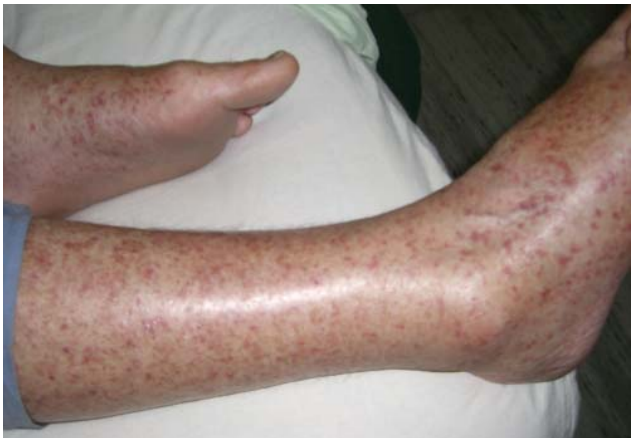

Figure. Rash exhibited by patient infected with *Rickettsia honei*, Nepal, 2009.

≥2-fold higher than for any other species (Table). These results and compatible clinical features confirmed the diagnosis of *R. honei* infection.

Conclusions

FISF was described in 1991 in Flinders Island (an island off the southeastern coast of Australia near Tasmania) and was similar to fever caused by an SFG rickettsia. In 1992, *Rickettsia honei* isolates were obtained from 2 patients with FISF. These isolates were characterized by using molecular methods and proposed as a new species in 1998 named *R. honei* (*2*,*3*); strain RB^T is the type strain. Thai tick typhus strain TT-118, isolated from a tick in 1962 in Thailand (*2*, *3*), was shown to be a strain of *R. honei*. *R. honei* strain marmionii was detected in 2007 (*7*); although the precise taxonomic position of this subspecies is unknown.

R. honei has been associated with various tick species (*3*), including *Ixodes granulatus* (Thailand) and *Haemaphysalis novaeguineae* (*7*) (mainland Australia) associated with rats, and *Aponoma hydrosauri* ticks (now *Bothriocroton hydrosauri*) (Flinders Island, Australia) associated with reptiles. One explanation for the uncommon distribution of *R. honei* is that ectoparasites associated with migrating birds that feed on local reptiles may transmit *R. honei* to reptile ticks (*3*).

Human cases of *R. honei* infection have been reported on Flinders Island and elsewhere in Australia (Tasmania, South Australia, Queensland, Torres Strait Islands) since 1991 (*8*) and in Thailand (*4*). Disease occurs primarily in spring and summer and has been mild; no deaths have been reported. Common features include fever, headache, myalgia, cough, arthralgia, and maculopapular to purpuric rash without vesiculation. An eschar is reported in 50% of cases (*2*,*3*).

Encephalitis, pneumonitis, tinnitus, and deafness in the patient are complications not reported with *R. honei* infections. Deafness has been reported with other SFG rickettsioses, particularly Rocky Mountain spotted fever (*9*) and infection with *O. tsutsugamushi* (*10*). Unsworth et al. reported 7 cases of FISF caused by *R. honei* strain marmionii that showed epidemiologic and clinical features different from those of classic FISF (*7*). Cases occurred in late summer and fall; cutaneous eschar was uncommon, and rash was not found on palms and soles of extremities (*7*). However, severe disease did not develop in any of these case-patients. The case we report differs from both patterns described. Unsworth et al. reported 1 chronic case of infection with *R. honei* strain marmionii, isolation of rickettsiae on day 27, and 1 patient with recrudescent

Table. Kinetics of species-specific antibody titers in patient infected with *Rickettsia honei*, Nepal, 2009*

	IgG/IgM titer by date			
Species tested	Apr 20	Apr 24	Apr 29	May 15
R. honei	Neg	256/32	512/32	1,024/64
R. felis	32/0	256/16	256/16	256/16
R. massiliae	Neg	Neg	Neg	128/16
R. aeschlimannii	Neg	Neg	Neg	128/16
R. conorii subsp. *israelensis*	Neg	Neg	Neg	128/16
R. conorii subsp. *conorii*	Neg	Neg	Neg	128/16
R. conorii subsp. *mongolitimonae*	Neg	Neg	Neg	128/18
R. slovaca	Neg	Neg	Neg	128/16
R. helijongangensis	Neg	32/32	64/32	64/64
R. AT1	Neg	32/32	64/32	64/64
R. africae	Neg	32/32	64/32	64/64
R. japonica	Neg	32/32	64/32	64/64
R. conorii subsp. *indica*	Neg	Neg	64/32	64/32
R. typhi	Neg	Neg	Neg	64/64
R. prowazekii	Neg	Neg	Neg	64/64
Orientia tsutsugamushi serotype Kawasaki	Neg	Neg	Neg	64/64
O. tsutsugamushi serotype Gilliam	Neg	Neg	Neg	Neg

*Ig, immunoglobulin; neg, negative. Titer cutoff values were ≥128 for IgG and ≥64 for IgM. A negative titer was reported when an initial serum screening result was negative. A titer of 0 was reported when an initial screening result was positive but no Ig was detected.

disease (day 33) after a 10-day symptom-free period (*11*). However, data from other studies, such as detection of *R. honei* in ticks in Texas (*3*) or in blood of patients without fever (*8*), could represent PCR contamination; these results need to be confirmed (*12*).

The case in this study provides evidence for human infection with SFG rickettsiosis in Nepal, where murine typhus and scrub typhus have only recently been described (*1*) and SFG rickettsioses have only been suspected (*13*). A preliminary study reported isolation of strain TT-118 from a *Rhipicephalus haemaphysaloides* ticks in Nepal (*14*). Moreover, *Ix. granulatus* ticks, which are associated with *R. honei* in Thailand, have been found in Nepal (*15*).

We propose that FISF may be an endemic rickettsiosis in Nepal and that studies of SFG *Rickettsia* spp., particularly *R. honei* in this region, are needed. Our report of *R. honei* infection in Nepal suggests a broader geographic distribution of FISF than believed. Clinical and entomologic research may improve our understanding of the etiology of febrile illness and the neglected field of emerging rickettsioses in Asia.

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Outbreak of West Nile Virus Infection in Greece, 2010

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During 2010, an outbreak of West Nile virus infection occurred in Greece. A total of 197 patients with neuroinvasive disease were reported, of whom 33 (17%) died. Advanced age and a history of heart disease were independently associated with death, emphasizing the need for prevention of this infection in persons with these risk factors.

An outbreak of West Nile virus (WNV) infection occurred in Central Macedonia in northern Greece in the summer of 2010. The first cases were diagnosed and reported to the Hellenic Centre for Disease Control and Prevention (HCDCP) on August 5, 2010 (*1*). WNV lineage 2 sequences were later obtained from 3 pools of *Culex pipiens* mosquitoes trapped at 2 sites where cases of West Nile neuroinvasive disease (WNND) had occurred (*2*).

Human cases of WNV disease had not been previously reported in Greece. Serosurveys in the early 1960s, 1980s, and 2007 identified WNV antibodies in ≈1% of the population, suggesting that WNV, or a related flavivirus, was circulating in Greece (*3*–*5*). In contrast, during 2005–2007, a total of 9,590 blood donors were tested by WNV nucleic acid amplification assay and results were negative (*6*).

The Study

After the outbreak alert was issued in early August 2010, physicians in Greece were asked to report all cases of WNV infection to HCDCP, according to the current European Union case definition (*1*). Only deaths that occurred during hospitalization were attributed to WNV infection. Statistical methods are described in the online Technical Appendix (www.cdc.gov/EID/content/17/10/11-0525-Techapp.pdf).

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Serum and cerebrospinal fluid specimens were tested for immunoglobulin (Ig) M and IgG against WNV by using an ELISA (WNV IgM capture DxSelect and WNV IgG DxSelect; Focus Diagnostics Inc., Cypress, CA, USA). Titers >640 were obtained by microneutralization assay against WNV in 14/14 patients who were positive for WNV. Although tick-borne encephalitis virus (TBEV) and dengue virus (DENV) are not prevalent in Greece, specimens were also tested for TBEV and DENV by ELISA (TBE/FSME IgM and TBE/FSME IgG; IBL International Gmbh, Hamburg, Germany) and Dengue Virus IgM Capture Dx Select and IgG Dx Select (Focus Diagnostics Inc.). All specimens were negative for TBEV, and cross-reactivity was seen with DENV, mainly for IgM (*7*).

Overall, 262 patients with WNV infection were reported to HCDCP. Of these patients, 197 (75%) had neuroinvasive disease (encephalitis, meningitis, or acute flaccid paralysis), and 65 (25%) had WNV fever. This study focused on patients with WNND, who were identified and reported more consistently because of disease severity.

Patient disease onset occurred within a 14-week interval during July 6–October 5, and the outbreak peaked in mid August (Figure 1). Most (94%) patients with WNND were reported from the 7 districts of Central Macedonia (Figure 2), and the epicenter of the outbreak was in Pella and Imathia Districts.

Characteristics of patients with WNND are shown in Table 1. Median age of patients with neuroinvasive disease was 72 years (range 12–88 years). The attack rate for WNND increased significantly (p = 0.006) with age (Table 1). The incidence of WNND in older persons (≥80 years of age) was ≈50× higher than that among the youngest age

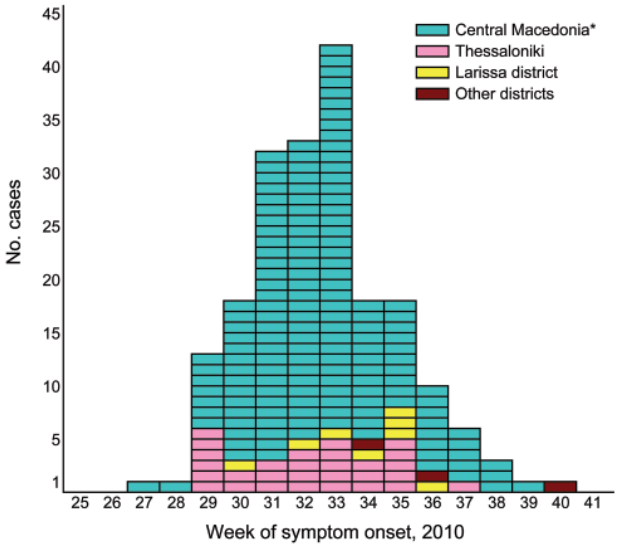

Figure 1. Reported cases (n = 197) of West Nile neuroinvasive disease, by week of symptom onset, Greece, July 6–October 5, 2010. *Excluding Thessaloniki.

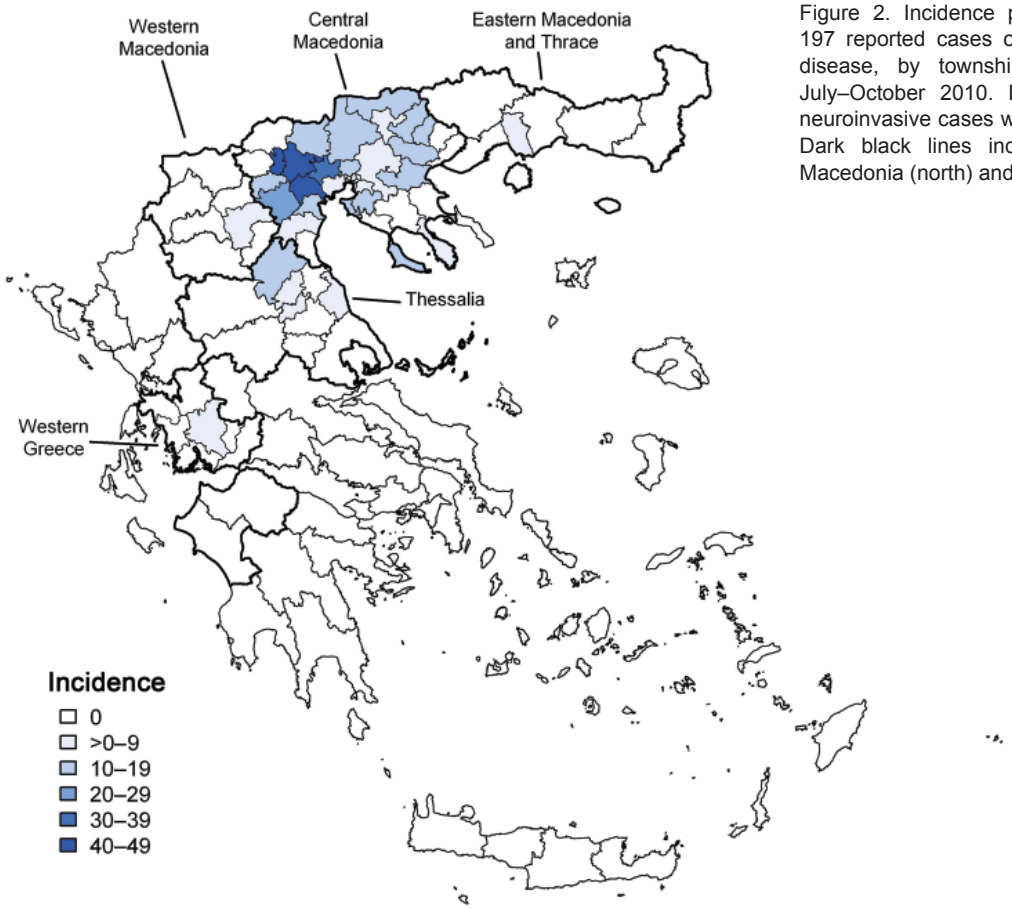

Figure 2. Incidence per 100,000 population of 197 reported cases of West Nile neuroinvasive disease, by township of residence, Greece, July–October 2010. Districts with ≥1 reported neuroinvasive cases were divided into townships. Dark black lines indicate borders of Central Macedonia (north) and Thessalia (south).

group (<20 years of age). Persons living in rural areas were 2× as likely to show development of WNND than persons living in urban areas (Table 1).

Encephalitis/meningoencephalitis (168 patients, 85%) was the most prominent clinical syndrome among patients with WNND, followed by meningitis (23, 12%). In addition, 10 (5%) patients with acute flaccid paralysis were reported, 6 (3%) of whom did not have meningitis or encephalitis.

A large proportion (74%) of patients with WNND had ≥1 underlying chronic medical condition; the most common were hypertension (39%), heart disease (24%), diabetes mellitus (24%), and immunosuppression (10%). Patients with WNND were 2× more likely (odds ratio 2.16, 95% confidence interval 1.15–4.04) than patients without WNND to have underlying conditions.

Thirty-five patients died during hospitalization (33 had WNND), indicating an overall case-fatality rate of 17% among persons with WNND. Median age of WNND patients who died was 78 years (range 49–87 years). The case-fatality rate increased substantially ($p<0.001$) with age (Table 2). Median interval from WNV disease onset to death was 13 days (range 3–90 days). In 15 (45%) patients with WNND who died, the interval between disease onset and death exceeded 2 weeks.

WNND patients with ≥1 underlying disease were 5× more likely to have died than patients without underlying conditions. Those patients who had heart disease or a stroke were ≈2.5× more likely to have died than patients without these conditions. However, only older age and heart disease were independent predictors of death in the final binomial regression model (Table 2). Supplementary results are shown in the online Technical Appendix.

Conclusions

Human cases of WNV infection were detected in several European and Mediterranean countries in 2010, indicating an increased intensity of viral circulation (*8*). Clinical cases of WNV infection in humans or animals had not been previously reported in Greece. The present outbreak was the largest in Europe since 1996, when a large outbreak was observed in Romania (*9*). The outbreak was located in Central Macedonia, which contains 90% of the rice paddies and 70% of the wetland areas in Greece and provides a favorable environment for reproduction of mosquito vectors (*8*). The region also hosts one of the largest populations of migratory birds in Greece. Meteorologic data for the area indicate that 2010 was warmer than previous years and unusually wet (*8*).

Table 1. Characteristics of 197 patients with West Nile neuroinvasive disease. Greece, July–October 2010*

Characteristic	No. patients	Incidence per 100,000 population	Risk ratio (95% CI)
Age group, y			
<20	4	0.18	Reference
20–29	3	0.20	1.08 (0.24–4.84)
30–39	6	0.34	1.87 (0.53–6.63)
40–49	9	0.55	3.03 (0.93–9.82)
50–59	18	1.27	6.93 (2.35–20.49)
60–69	29	2.44	13.31 (4.68–37.84)
70–79	85	8.01	43.74 (16.05–119.2)
≥80	43	9.63	52.62 (18.89–146.6)
Sex			
F	88	1.59	Reference
M	109	1.97	1.26 (0.95–1.67)
Place of residence			
Urban	110	1.38	Reference
Rural	87	2.92	2.12 (1.60–2.80)
Districts in Central Macedonia			
Chalkidiki	4	3.99	0.76 (0.28– 2.09)
Thessaloniki	60	5.27	Reference
Pieria	9	7.02	1.33 (0.66–2.69)
Serres	21	11.15	2.12 (1.29–3.48)
Kilkis	12	13.92	2.64 (1.42–4.91)
Imathia	39	27.06	5.14 (3.43–7.69)
Pella	41	28.26	5.37 (3.61–7.98)
Total	186	15.00	NA
Other districts (region)			
Etoloakarnania (western Greece)	1	0.46	0.09 (0.01–0.63)
Kozani (Western Macedonia)	1	0.65	0.12 (0.02–0.89)
Kavala (Eastern Macedonia)	1	0.71	0.14 (0.02–0.98)
Larissa (Thessalia)	8	2.80	0.53 (0.25–1.11)
Total in Greece	197	1.76	NA

*Incidence rates were calculated by using 2008 mid-year population estimates of the Hellenic Statistical Authority as the denominator. CI, confidence interval; NA, not applicable.

The overall case-fatality rate among patients with WNND (17%) was higher in Greece than that in other countries (*9*–*11*). The reasons for this finding are not clear. Many factors may have played a role in differences in the fatality rate. These factors include diagnosis and surveillance bias for more severe cases, virus strain, host susceptibility, age structure of the population, and underlying conditions.

Recent studies on WNV lineage 2 suggested that this virus may be underestimated as a cause of neuroinvasive disease (*2*,*12*). WNV linage 2 isolated from *Cx. pipiens* mosquitoes in the affected areas during this outbreak had a nucleotide genetic similarity of 99.6% with the goshawk Hungary 2004 strain (*12*). However, few severe cases of WNV infection were reported in Hungary. Experimental studies would verify whether the amino acid substitution H249P detected in the Greek strain, which is a suspected virulence marker in lineage 1 strains, is associated with increased virulence (*12*).

Advanced age and heart disease were found to independently predict the risk for WNND-related death. The association between age and severe disease has been reported (*9*–*11*). The contributing factor of age may relate to a decrease in the integrity of the blood–brain barrier and facilitate access of WNV to the central nervous system (*13*). Heart disease, particularly cardiac arrhythmias, have also been recognized as frequent contributors to death caused by WNV encephalitis (*13*–*15*). Physiologic stress of WNV infection may precipitate or exacerbate underlying medical conditions resulting in death (*14*).

These findings emphasize the need for primary prevention of WNV infection in patients with these predisposing conditions and close monitoring for cardiac complications in elderly patients hospitalized with WNV disease. Vector mosquito control programs, including source reduction and larviciding of *Culex* spp. mosquitoes and ongoing public health education and WNV surveillance in disease-endemic and newly affected areas, remain the cornerstones of WNV disease prevention and control.

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Table 2. Predictive factors of death for 197 patients with West Nile neuroinvasive disease analyzed by univariate and multivariate analysis, Greece, July–October 2010*

Characteristic	No. deaths, n = 33†	Case-fatality rate, %	Crude risk ratio (95% CI)	Adjusted risk ratio‡ (95% CI)
Age group, y				
40–59	1§	2.50	Reference	Reference
60–69	2	6.90	2.76 (0.26–28.99)	2.72 (0.26–28.40)
70–79	15	17.65	7.06 (0.97–51.59)	6.13 (0.83–45.17)
≥80	15	34.88	13.95 (1.93–100.9)	11.41 (1.56–83.52)
Sex				
F	10	11.36	Reference	NA
M	23	21.10	1.86 (0.93–3.69)	NA
Underlying diseases				
None	2	3.92	Reference	NA
≥1	31	21.23	5.41 (1.34–21.82)	NA
Hypertension				
No	19	15.70	Reference	NA
Yes	14	18.42	1.17 (0.63–2.20)	NA
Heart disease				
No	18	12.00	Reference	Reference
Yes	15	32.61	2.72 (1.49–4.95)	2.03 (1.14–3.64)
Diabetes				
No	23	15.44	Reference	NA
Yes	10	20.83	1.35 (0.69–2.63)	NA
Immunosuppression				
No	31	17.42	Reference	NA
Yes	2	10.53	0.60 (0.16–2.33)	NA
Cancer				
No	29	16.11	Reference	NA
Yes	4	23.53	1.46 (0.58–3.66)	NA
Stroke				
No	28	15.30	Reference	NA
Yes	5	35.71	2.33 (1.07–5.10)	NA
Renal failure				
No	31	16.40	Reference	NA
Yes	2	25.00	1.52 (0.44–5.28)	NA

*CI, confidence interval; NA, not applicable.
†Two additional patients with nonneuroinvasive disease died, and those deaths were not included in this analysis.
‡In logistic regression analysis, initial models included all variables for which the p value was <0.05 or the odds ratio was >1.1 or <0.90. Therefore, all variables were included in the initial models. Variables were removed 1 at a time depending on results of statistical testing (p<0.05), by using the likelihood-ratio test. All variables that remained significant in the final logistic regression model were included in the binomial regression model for the estimation of adjusted risk ratios.
§Belonged to the 40–49-year age group.

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Tembusu Virus in Ducks, China

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In China in 2010, a disease outbreak in egg-laying ducks was associated with a flavivirus. The virus was isolated and partially sequenced. The isolate exhibited 87%–91% identity with strains of Tembusu virus, a mosquito-borne flavivirus of the Ntaya virus group. These findings demonstrate emergence of Tembusu virus in ducks.

From June through November 2010 in the People's Republic of China, a disease characterized by a sudden onset was observed on many egg-laying and breeder duck farms. Egg production in affected ducks dropped severely within 1–2 weeks after disease onset. Other consistent signs included acute anorexia, antisocial behavior, rhinorrhea, diarrhea, ataxia, and paralysis. Rate of illness was usually high (up to 90%), and mortality rates varied from 5% to 30%. From affected ducks we isolated and identified a Tembusu virus (TMUV).

The Study

During the outbreak, we examined 11 diseased ducks (7 Pekin ducks, 3 Cherry Valley Pekin ducks, and 1 Shaoxing duck) from 5 duck farms in 4 provinces. At necropsy, viscera samples (e.g., brain, heart, liver, spleen, lung, theca folliculi) were collected and placed in 10% buffered formalin. Sections were embedded in paraffin and stained with hematoxylin and eosin. The theca folliculi from each duck were also used for virus isolation or detection by PCR.

The main pathologic changes observed consistently in almost all diseased ducks were found in the ovaries: hyperemia, hemorrhage, degeneration, distortion, macrophage and lymphocyte infiltration, and hyperplasia; in the liver, interstitial inflammation was found in the portal area (Figure l, panels A–C). On the basis of these changes, the disease was designated duck hemorrhagic ovaritis.

After samples underwent several rounds of screening and identification attempts, we concluded that a new virus infection was the most likely cause of the disease. One virus strain, designated YY5, was isolated from the Shaoxing duck after inoculation of clarified theca folliculus suspension into the allantoic cavities of 9-day-old specific pathogen–free chicken embryos. The embryos died 72–120 hours after inoculation, and severe cutaneous hemorrhages were observed.

Because eastern equine encephalitis (EEE) virus has been shown to cause paralysis in Pekin ducks (*1*), we performed EEE virus–specific nested reverse transcription PCR (RT-PCR) (*2*) to detect the isolate. RNA was extracted by using the TranZol RNA Extraction Kit (TransGen Biotech, Beijing, China). The primer pairs used to amplify the E2 gene of EEE virus (EEE-4 and cEEE-7, EEE-5 and cEEE-6) have been described (*2*). A clear PCR product (268 bp) resulted and was then sequenced; the deduced amino acid sequence was compared with other sequences by using a BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search in GenBank. Unexpectedly, a 221-nt sequence (GenBank accession no. HQ641388) was shown to encode the nonstructural (NS) 1 protein of flavivirus, which exhibited 73%–85% identity to flaviviruses in the Ntaya virus and Japanese encephalitis virus groups, such as Bagaza virus (GenBank accession no. ACG60714, [*3*]) and St. Louis encephalitis virus (GenBank accession no. ABN11829 [*4*]). The result demonstrated the possible presence of a flavivirus in ducks.

To further confirm flavivirus as the causative agent of duck hemorrhagic ovaritis, we used PCR to test the isolate and clinical samples with forward primer Usu5454f (5′-ATGGATGAAGCYCATTTCAC-3′) (*5*) and a newly designed reverse primer 5861R (5′-CCAAAGTTGGCYCCCATCTC-3′). The primers were located in the conserved regions of the NS3 sequences of Bagaza virus, St. Louis encephalitis virus, and Usutu virus (*3*–*5*) and were predicted to produce an ≈400-bp amplicon. The reaction conditions were as follows: 5 min at 94°C; followed by 38 cycles of denaturation at 94°C for 40 s, annealing at 47°C for 35 s, and extension at 72°C for 1 min; and a final extension of 72°C for 10 min. RT-PCR

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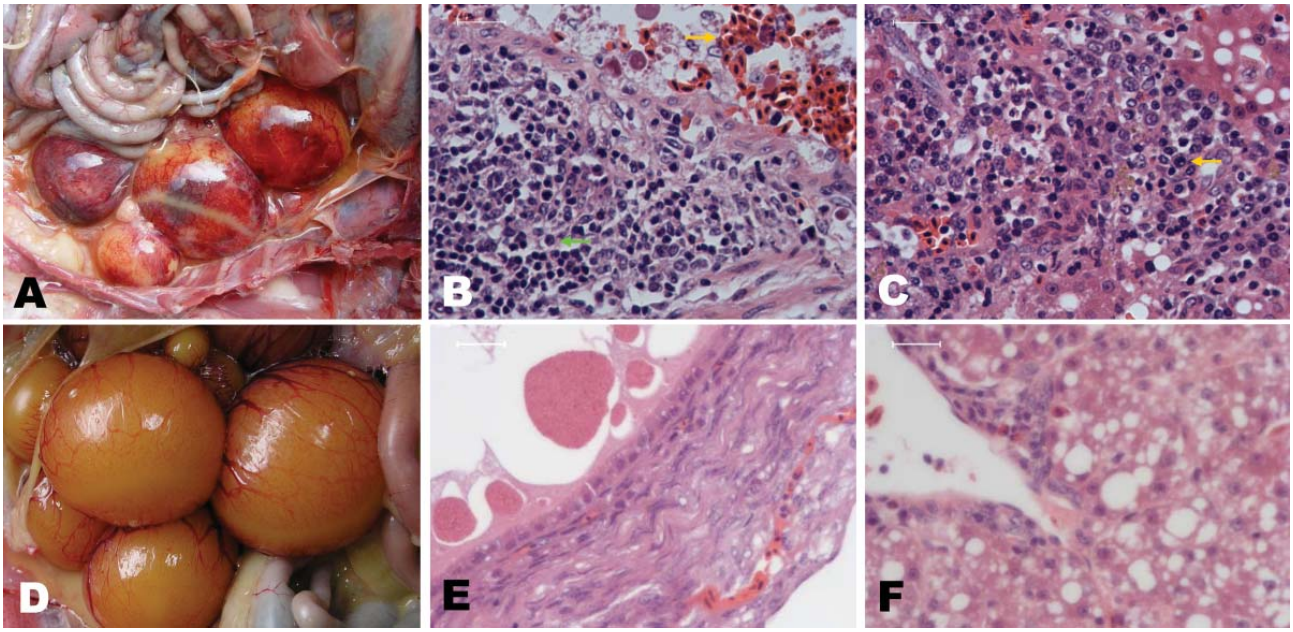

Figure 1. Pathologic changes in diseased Pekin ducks. A) Ovary with hyperemia, hemorrhage, and distortion. B) Ovary with hemorrhage (gold arrow), macrophage and lymphocyte infiltration and hyperplasia (green arrow). C) Liver with interstitial inflammation in the portal area (gold arrow). D and E) Ovaries from healthy ducks. F) Liver from healthy duck. A, C) Original magnification ×40; B, C, E, F) scale bars = 90 μm.

was optimized by using the following controls, including nucleic acids extracted from theca folliculi of healthy Pekin ducks: avian influenza virus, Newcastle disease virus, egg drop syndrome virus, anatid herpesvirus 1, Muscovy duck parvovirus, goose parvovirus, duck reovirus, goose reovirus, duck hepatitis A virus, duck astrovirus, duck circovirus, and goose hemorrhagic polyomavirus.

All 11 theca folliculus samples and the isolate were positive for flavivirus by RT-PCR, which was confirmed by amplicon sequencing. The 367-nt sequence (GenBank accession no. HQ641389) of part of the NS3 genomic region obtained from the isolate was 66%–77% identical to the corresponding sequence of viruses in the Ntaya virus and Japanese encephalitis virus groups. The amplicon sequences from the 11 theca folliculus samples shared 98%–100% identity with the YY5 isolate. PCR testing of another 52 samples from diseased ducks from the 4 provinces detected flavivirus-specific RNA in 29 samples. Overall, 40 (63.5%) of 63 samples were positive for flavivirus. Flavivirus-specific RNA was most frequently detected in theca folliculi, followed by intestinal mucosa, uterus, spleen, trachea, cloaca (swab), and liver (Table).

Table. Flavivirus detection in 63 samples from diseased ducks, 4 provinces, People's Republic of China, 2010

Sample	No. (%) flavivirus positive	No. flavivirus negative
Theca folliculi, n = 15	14 (93.3)	1
Intestinal mucosa, n = 4	3 (75.0)	1
Uterus, n = 7	5 (71.4)	2
Spleen, n = 9	6 (66.7)	3
Trachea, n = 2	1 (50.0)	1
Cloacal swab, n = 17	8 (47.1)	9
Liver, n = 9	3 (33.3)	6

Subsequently, the YY5 isolate was injected intramuscularly into nine 55-week-old Pekin ducks and ten 30-week-old Shaoxing ducks. At day 4 postinoculation, the pathologic changes were reproduced in these experimentally infected ducks. The flavivirus RNA was detected by NS3-based RT-PCR, and the virus was again isolated from theca folliculi.

To investigate the genetic relationship of the isolate with flaviviruses, we obtained the genomic sequence of a 1,035-bp segment at the 3′ terminus of the NS5 gene (GenBank accession no. HQ641390) from strain YY5 by RT-PCR and primers FU1 and cFD3 as described (*6*). Phylogenetic analysis showed that YY5 was more closely related to TMUV than to other flaviviruses (Figure 2). Comparative sequence analysis showed that YY5 was 87%–91% identical to different strains of TMUV; therefore, we classified the flavivirus isolated from ducks as a new genotype of TMUV, a mosquito-borne flavivirus of the Ntaya virus group.

Conclusions

We have demonstrated the presence of a mosquito-borne flavivirus in ducks. On the basis of criteria for species of the members of the genus *Flavivirus* (*6*) and phylogenetic analysis, we consider the isolate to belong to a new genotype of TMUV.

In this study, we found TMUV-specific RNA in 63.5% samples from diseased ducks in different provinces. In particular, it was found in 14 (93.3%) of 15 theca folliculus samples, suggesting that reproductive tissues may be a major site for viral persistence, replication, or both. Experimental infections further confirmed that TMUV can be reisolated from theca folliculi. These results suggested that TMUV may be the causative agent of duck hemorrhagic ovaritis.

Because TMUV belongs to the mosquito-borne virus cluster of flaviviruses, mosquitoes might be involved in the spread of this virus. Detection of the virus in cloacal swab samples suggests probable horizontal transmission through ingestion or inhalation of feces-contaminated material.

TMUV was originally isolated from mosquitoes of the genus *Culex*, but the disease associated with TMUV infection was not known. However, a chick-origin TMUV isolate, originally named Sitiawan virus, can cause encephalitis and retarded growth in broiler chicks (*8*). In conclusion, this study shows that duck-origin TMUV is highly pathogenic for Pekin ducks, Cherry Valley Pekin ducks, and Shaoxing ducks.

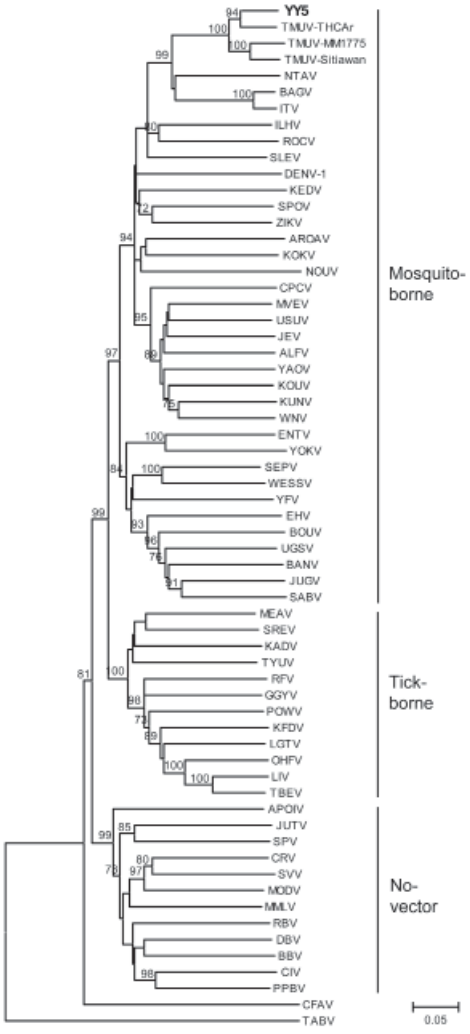

Figure 2. Phylogenetic analysis of isolate YY5 (in **boldface**) from an ill Shaoxing duck in the People's Republic of China and selected other flaviviruses obtained by using an ≈1-kb nt sequence in the nonstructural 5 genomic region. The tree was constructed by the neighbor-joining method of MEGA (*7*). Numbers at nodes indicate bootstrap percentages obtained after 1,000 replicates; only bootstrap values >70% are shown. Scale bar indicates genetic distance. The sequences used in the phylogenetic analysis are listed online (www.cdc.gov/EID/content/17/10/10-1890-F.htm). The nucleotide sequence of isolate YY5 used in the phylogenetic analysis has been deposited in GenBank under accession no. HQ641390.

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Novel Amdovirus in Gray Foxes

Linlin Li, Patricia A. Pesavento, Leslie Woods, Deana L. Clifford, Jennifer Luff, Chunlin Wang, and Eric Delwart

We used viral metagenomics to identify a novel parvovirus in tissues of a gray fox (*Urocyon cinereoargenteus*). Nearly full genome characterization and phylogenetic analyses showed this parvovirus (provisionally named gray fox amdovirus) to be distantly related to Aleutian mink disease virus, representing the second viral species in the *Amdovirus* genus.

Aleutian mink disease virus (AMDV) is currently the only member of the genus *Amdovirus* in the family *Parvoviridae*; it can infect diverse breeds of farmed and feral mink, in addition to other mustelids (e.g., ferrets, otters), raccoons, and foxes (*1*,*2*). AMDV has an ≈5-kb single-stranded DNA genome and, like other parvoviruses, replicates through a rolling-hairpin mechanism (*3*). The viral genome has 2 large open reading frames (ORFs), encoding nonstructural (NS1, NS2, putative NS3) and structural viral proteins (VP1 and VP2). Alternative splicing enables expression of multiple messenger RNAs (*4*). AMDV strains can exhibit sequence variability in their NS gene, and 3 genetic groups have been identified on the basis of partial nucleotide sequences of this region (*5*).

AMDV infection can cause an acute and fatal interstitial pneumonia in newborn mink. It can also cause a chronic disorder of the immune system in adult mink, characterized by persistent viral infection, plasmacytosis, hypergammaglobulinemia, and immune complex–mediated glomerulonephritis and arteritis, resulting in major economic losses to mink farms (*6*). AMDV infection can also be asymptomatic. The different outcomes are determined by host factors that include age, immune status, and the virulence of the virus strains (*7*,*8*). AMDV can be transmitted through urine, feces, and saliva as well as vertically in utero (*9*,*10*). In 1 report, a ferret found to be naturally infected with AMDV showed acute dyspnea and posterior paresis with histopathologic lesions similar to those seen in mink; the ferret became comatose and died (*11*). Recently, 2 mink farmers with vascular disease and microangiopathy, similar to conditions in mink with Aleutian disease, were found to have AMDV-specific antibodies and were AMDV DNA positive, suggesting a potential relationship between AMDV and human symptoms (*12*).

We used random PCR amplification and high-throughput sequencing technology to investigate viral sequences found in the spleen and lung tissues of a sick gray fox (*Urocyon cinereoargenteus*) from California. A highly divergent amdovirus was identified, and the near full genome of this virus was obtained. Phylogenetic analysis indicated that this virus, designated as gray fox amdovirus, is a new amdovirus species, only the second for that genus.

The Study

The gray fox studied here was identified during the summer of 2009 in Sonoma County, California. It had severe gait abnormalities, lymphadenopathy, and acute muscle inflammation, and was euthanized at a wildlife rehabilitation center. Using the generic viral particle enrichment method previously described for tissues (*13*), we generated ≈14,000 sequence reads from spleen and lung samples. We found 136 sequence reads in spleen tissue that were related to AMDV by using BLASTx (E score $<10^{-5}$) (www.ncbi.nlm.nih.gov/BLAST); these could be assembled into 24 contigs covering ≈60% of the viral genome. By connecting gaps between sequenced viral fragments and amplifying the genome extremities by using PCR primers based on AMDV sequences, the nearly complete genome of the new amdovirus (GenBank accession no. JN202450) was acquired. We temporarily named it gray fox amdovirus (GFADV).

The partial GFADV genome was 4,441 nt in length with a low guanine–cytosine content of 37%. Similar to that of AMDV, the GFADV genome contained 2 major ORFs. The left ORF (LORF) contains the bulk of the sequences for the putative NS1, and the right ORF (RORF) codes for VP2 (Figure 1). Two small middle ORFs (67 and 75 aa long) with putative alternative start codons were detected in the 448-bp region between the ORFs. The theoretical proteins showed 55% and 59% aa identity with the 2 similarly located middle ORFs reported in AMDV. The partial 5′ untranslated region (UTR) was 109 nt and the partial 3′ UTR was 191 nt. Potential RNA splicing signals in AMDV were present on the GFADV genome (online Technical Appendix Figure 1, www.cdc.gov/EID/content/17/10/11-0233-Techapp.pdf) (*4*). The predicted spliced transcripts encode hypothetical NS1, NS2, and NS3 of 635 aa, 115 aa, and 80 aa, respectively, and a capsid protein VP1 of 674 aa. The putative VP2

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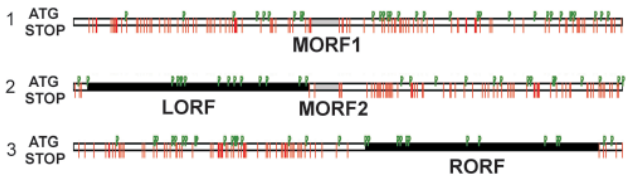

Figure 1. Open reading frames (ORFs) in gray fox amdovirus genome. Three possible reading frames of the plus-strand sequence with the stop codons indicated by red lines and ATG codons by green flags. Two major ORFs, left (LORF) and right (RORF), are indicated by black bars; 2 small middle ORFs (MORF1 and MORF2) are indicated by gray bars.

was predicted to arise from the intact transcript of RORF encoding a 630-aa protein.

Sequence analyses confirmed that GFADV was a divergent amdovirus with 76% nt identity with the genome of AMDV. Conserved protein domains typical of parvoviruses were identified in GFADV. In the LORF, the GKRN domain was found (online Technical Appendix Figure 2), which may act as the nuclear transport signal of NS1 protein. In the RORF, 3 conserved domains (TPW, YNN, and PIW) of unknown biologic significance were detected (*6*,*14*) (online Technical Appendix Figure 3). The phosholipase 2 motif in the N terminal VP1 region, generally conserved in parvoviruses, was not found in either GFADV not AMDV (online Appendix Figure 3), which suggests that this parvovirus genus uses a different mechanism to escape the endosome during infection (*15*).

Comparison of NS1 regions showed GFADV shared ≈74% nt and 67% aa similarities with AMDV strains, whereas different strains of AMDV shared >87% nt and 82% aa similarities. Alignments of the VP2 region showed GFADV shared ≈78% nt and 80% aa similarities with AMDV strains, whereas strains of AMDV had >92% nt and 91% aa similarities (online Technical Appendix Figures 4, 5). To determine the relationship between GFADV and AMDV strains, phylogenetic analyses of the NS1 and VP2 proteins were performed, which showed that in both genome regions GFADV was more closely related to AMDV strains than to those of minute virus of mice or other parvoviruses analyzed (data not shown), but was distinct from the 3 AMDV groups (Figure 2). Pending review by the International Committee on Taxonomy of Viruses, GFADV thus appears to be the second reported parvovirus species in the genus *Amdovirus*.

GFADV sequences were also detected in the lung and heart tissues of the same animal by using a GFADV-specific nested PCR targeting a ≈400-bp segment of the VP2 gene, as well as in the heart tissue of another gray fox, which had the same signs, collected in Sonoma County in summer 2009. Further PCR screening of 19 tissue samples, including spleen, lung, liver, lymph node, and muscle from 9 other gray foxes with similar gait abnormalities and chronic muscle lesions, collected in 2008 (n = 2) and 2010 (n = 7) were negative by the same GFADV PCR.

Conclusions

We report the identification and nearly complete genome sequence of an amdovirus found in the spleen, lung, and heart tissues of 2 gray foxes that exhibited an abnormal gait and muscle inflammation of unknown origin. On the basis of phylogenetic analyses, we propose this virus as the prototype member of a second species in the *Parvoviridae* genus *Amdovirus*. Putative NS and VP1/VP2 gene RNA splicing sites were detected in the GFADV genome, which suggests the expression of different NS and VP proteins.

Except for the ubiquitous anellovirus, GFADV was the only eukaryotic virus found in the spleen tissue of the diseased gray fox. The same virus was also identified in the heart tissue of a second gray fox collected the same summer but was not detected in tissues of 9 other gray foxes with a similar syndrome collected in different years. It is possible that GFADV is an incidental finding unrelated to these foxes' symptoms. The lack of detectable GFADV DNA in all gray foxes with similar symptoms may also be because different tissues were compared in some animals or because tissue collection occurred at different stage of infection. Future testing of a possible link between GFADV and additional unexplained diseases of foxes and

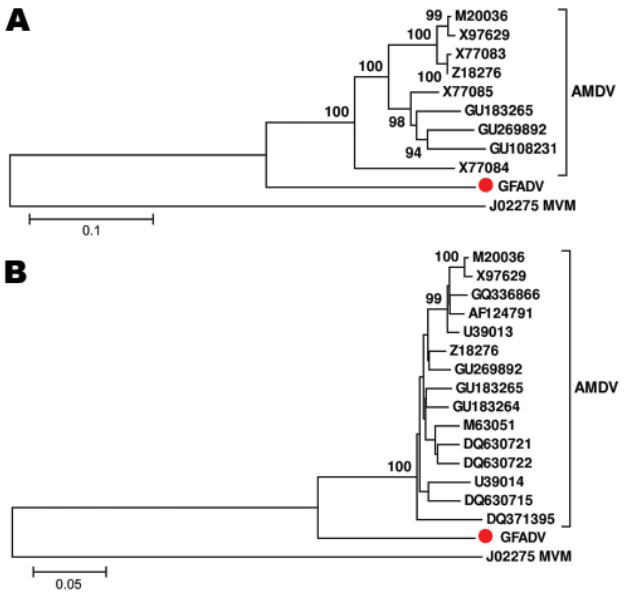

Figure 2. Phylogenetic analyses of gray fox amdovirus (GFADV) (red dots) and Aleutian mink disease virus (AMDV) based on the complete amino acid sequence of nonstructural protein 1 region (A) and viral protein 1 region (B). The neighbor-joining method was used with *p*-distance and 1,000 bootstrap replicates. Scale bars represent estimated phylogenetic divergence. GenBank accession numbers are shown on the tree. Minute virus of mice (MVM) was included as an outgroup.

other carnivores will be facilitated by the availability of its genome sequence.

Acknowledgments

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Dr Li is a staff scientist at the Blood Systems Research Institute, San Francisco, California. Her research interests are infectious diseases and viral discovery.

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Bacteremia and Antimicrobial Drug Resistance over Time, Ghana

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Bacterial distribution and antimicrobial drug resistance were monitored in patients with bacterial bloodstream infections in rural hospitals in Ghana. In 2001–2002 and in 2009, *Salmonella enterica* serovar Typhi was the most prevalent pathogen. Although most *S. enterica* serovar Typhi isolates were chloramphenicol resistant, all isolates tested were susceptible to ciprofloxacin.

In Africa, fever is usually a synonym for malaria. However, evidence exists that a large proportion of fever of unknown origin (FUO) can be attributed to bacterial bloodstream infections (BBSI). Although *Staphylococcus aureus* is the predominant cause of BBSI in industrialized countries (*1*), in African countries such as Ghana or Kenya, gram-negative bacteria are identified most often in BBSI (*2*,*3*). Furthermore, because of a lack of epidemiologic data, FUO in Africa is often treated sequentially, first with antimalarial drugs and then, until some years ago, with antimicrobial drugs such as chloramphenicol. This strategy has often been ineffective (*4*).

The Study

In 2000 in hospitals in Ghana, we began to establish bacteriologic laboratories, which since then have participated in a biannual quality control program. For this quality control, 3 encoded bacterial species and their resistance to various antimicrobial drugs must be correctly identified. Three of these hospitals took part in comparative epidemiologic studies of FUO during October 2001–April 2002 and again during August–September 2009 with the objective of establishing a rational treatment approach (Figure). The hospitals were located in Eikwe, a coastal village that has a rural population of ≈2,000 residents; Assin Foso, which is on a regional traffic route and has a rural/urban population of ≈15,000 residents; and Nkawkaw, which is on the national traffic route that connects Accra with Kumasi and has an urban population of >45,000 residents.

This study was approved by the ethical committee of the University Medical Center, Göttingen, Germany, and the participating hospitals in Ghana. The study design, patient selection, and diagnostic approaches were identical in both study periods; FUO was defined as fever >38.5°C of >1 week's duration without a clear clinical or organ-specific diagnosis. During the first study period, 409 patients with a wide range of ages (interquartile range 26 years) were investigated. The second study period included 258 patients with a similar age distribution (interquartile range 27 years).

Blood film microscopy was used for malaria diagnosis. Bacteremia was determined by blood cultures; 2 mL or 5 mL of blood was incubated in 20 mL or 50 mL of locally

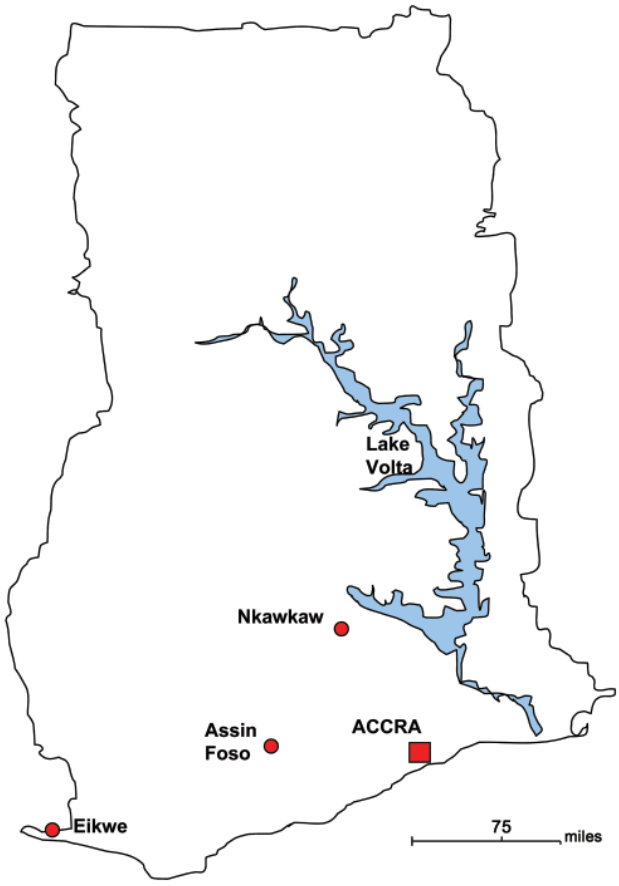

Figure. Location of populations in a study of bacteremia and antimicrobial drug resistance over time, Ghana.

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made brain–heart infusion broth for ≤7 days at 37°C. Gram stains and subcultures on chocolate agar were performed after 24 h, 72 h, or when the media became turbid. Bacterial differentiation, according to good laboratory practice, and susceptibility testing by disk diffusion following National Committee for Clinical Laboratory Standards criteria (*5*) was done in Africa, and susceptibility testing that included quinolone susceptibility was confirmed by broth microdilution at the University Medical Center, Göttingen. Respective tests for species differentiation were also repeated in Göttingen. The Vi phage typing scheme from the Colindale Institute London was used for *Salmonella* spp. typing (*6*).

Of the 212 bacterial isolates recovered from the blood cultures in the first study period, 145 (68.4%) indicated a putative agent of septicemia (Table 1). *Salmonella enterica* was identified in 100 (69.0%) of all pathogen-positive blood cultures, with *S. enterica* serovar Typhi accounting for 59 (40.7%). Although the 2001 National Guidelines of Ghana listed chloramphenicol as first choice for treating typhoid fever, >80% of all bacteria identified (88.3% of all *S. enteric* serovar Typhi) were resistant to this drug. However, ciprofloxacin proved effective against most bacteria, especially against *S. enterica* serovar Typhi (Table 2). Thus, in 2004, the national guidelines replaced chloramphenicol with ciprofloxacin for treating typhoid fever (*7*).

To analyze the influence of ciprofloxacin on pathogen distribution and antimicrobial drug resistance in BBSI, in 2009 we initiated a follow-up study. During the second study period, pathogenic bacteria were identified in 48 (48.5%) of 99 blood cultures; the rate of *Plasmodium*-positive patients was significantly higher (42.4% vs. 24.0%, p<0.0001; Table 1). *S. enterica* was found in 50% (24/48) of all pathogen-positive blood cultures with *S. enterica* serovar Typhi remaining the most prevalent species (Table 1). Sampling was done during different months in the 2 study periods; however, these covered mainly the dry seasons. Although seasonal differences might have had an effect on the pathogen distribution, our finding is in accordance with results from other tropical countries (*8*,*9*). In addition, although our study regions were 75–150 miles away from each other, and the hospitals were localized in villages or cities which differ notably with regards to population and structure, most *S. enterica* serovar Typhi isolates belonged to phage type D1. Therefore, the spread of a clonal bacterial population within Ghana cannot be discounted.

Although an extraordinarily high percentage of chloramphenicol resistance was obvious, this drug still was considered the first choice treatment for typhoid fever in 2001 in Ghana. Therefore, the high rate of *S. enterica* serovar Typhi was not unexpected. Similarly, 91.7% of all *S. enterica* were resistant to chloramphenicol in 2009 (Table 2). In both study periods, second-line antimicrobial agents, e.g., trimethoprim/sulfamethoxazole or ampicillin, also showed a high rate of resistance. This finding was in agreement with those of other studies from nonindustrialized countries (*10*). The rate of cefuroxime-resistant bacteria increased from 18.9% to 41.7% because of a higher percentage of cefuroxime-resistant enterobacteriaceae other than *Salmonella* (50.0% vs. 87.5%, Table 2).

In 2001–2002, most bacteria were susceptible to ciprofloxacin (Table 2), as had been shown for *S. enterica* from blood cultures of Nigerian patients (*11*). In contrast,

Table 1. Comparative monitoring of bloodstream infections, Ghana, 2001–2002 and 2009*

Variable	July 2001–April 2002		July–September 2009	
	Total	Positive for *Plasmodium* spp.	Total	Positive for *Plasmodium* spp.
No. patients with fever of unknown origin	409	NA	258	NA
No. *Plasmodium* spp. positive/total no. tested (%)†	85/354 (24.0)	NA	75/177 (42.4)	NA
Total no. bacterial isolates	212	51 (24.1)	99	14 (14.1)
Skin flora contaminants	67	24 (35.8)	51	10 (19.6)
Potential pathogens	145 (100.0)	27 (18.6)	48 (100.0)	4 (8.3)
Salmonella enterica serovars	100 (69.0)	20 (20.0)	24 (50.0)	4 (16.7)
Typhi	59 (40.7)	11 (18.6)	15 (31.3)	2 (13.3)
Paratyphi	1 (0.7)	NF	0	NF
Nontyphoid‡	40 (27.6)	9 (22.5)	9 (18.8)	2 (22.2)
Staphylococcus aureus	16 (11.0)	3 (18.8)	3 (6.3)	NF
Enterobacteriaceae other than *Salmonella* spp.	10 (6.9)	1 (10.0)	8 (16.7)	NF
Pseudomonas spp.	7 (4.8)	2 (28.6)	5 (10.4)	NF
Other§	12 (8.3)	1 (8.3)	8 (16.7)	NF

*Values are no. (%) except as indicated. The total number of potential pathogens for each study period was set as 100%. Coagulase-negative staphylococci, micrococci, and bacilli were judged as skin flora contaminants. The ratio of *Plasmodium* spp.–positive patients in regard to bacterial species respective groups is indicated. NA, not applicable; NF, *Plasmodium* spp. not found.
†Blood film for *Plasmodium* spp. was done in most cases. If the clinical situation, patient history, or blood count strongly indicated bacterial infection, blood culture was taken as first diagnostic approach.
‡*S. enterica* serovar Enteritidis and serovar Typhimurium.
§Including *Streptococcus* spp., *Enterococcus* spp., *Acinetobacter* spp.

Table 2. Ratio in percentages of antimicrobial drug–resistant bacterial isolates obtained from patients with bacterial bloodstream infections, Ghana, 2001–2002 and 2009*

Bacteria type and years	PEN	OXA	AMP	CEF	GEN	SMX	CMP	CIP
Salmonella enterica serovar Typhi								
2001–2002			93.3	1.7	0	86.7	88.3	0
2009			100	0	0	100	100	0
Nontyphoid *Salmonella* spp.								
2001–2002			100	20.0	12.5	90.0	82.5	0
2009			100	0	0	88.9	77.8	0
Enterobacteriaceae other than *Salmonella* spp.								
2001–2002			100	50.0	60.0	80.0	80.0	0
2009			100	87.5	37.5	62.5	50.0	50.0
Nonfermenters								
2001–2002			91.7	75.0	16.7	41.7	100	0
2009			100	100	15.4	53.8	92.3	0
Staphylococcus aureus								
2001–2002	81.3	0	81.3		0	0	68.8	
2009	100	0	100		0	0		
All bacteria								
2001–2002			93.6	18.9	10.7	72.1	84.3	0
2009			100	41.7	10.4	72.9	84.4	8.9

*Blank cells indicate no testing performed. PEN, penicillin; OXA, oxacillin; AMP, ampicillin; CEF, cefuroxime; GEN, gentamicin; SMX, trimethoprim/sulfamethoxazole; CMP, chloramphenicol; CIP, ciprofloxacin.

for *S. enterica* serovar Typhi isolated in 1997–1999 in Kenya, MICs of ciprofloxacin were noticeably higher than for those strains isolated during 1988–1993 (*12*,*13*).

Although ciprofloxacin proved to be effective against *S. enterica* in our study, the resistance rate of enterobacteriaceae other than *S. enterica* against this quinolone increased from zero in 2001–2001 to 50.0% in 2009 (Table 2). Methicillin-resistant *S. aureus* was not identified as a cause of BBSI during either period.

When we assessed the situation in individual regions, notable differences were obvious. Comprising 47.5% of all BBSI, typhoid fever was most prevalent in Assin Foso in 2001–2002. In contrast, not even 1 case occurred in 2009. Analyzing the situation in that urban area, the following conditions were found: 1) sanitation was improved; 2) additional toilets were established; 3) ciprofloxacin was widely used in hospital for treating infections; and 4) ciprofloxacin was easily available at local street traders. Although the broad application of ciprofloxacin has to be critically discussed, the observed absence of typhoid fever in Assin Foso is impressive.

In contrast, *S. enterica* serovar Typhi was isolated from 27.8% of cases of BBSI in Eikwe in 2001–2002 and remained at a high rate of 30.2% in 2009. In this small fishing village, the situation differed noticeably from that in Assin Foso. Although additional toilets had been constructed, sanitation was not much improved; most residents still used the beach for defecation. In addition, ciprofloxacin was not extensively prescribed in the hospital and was not available at local street traders. Thus, educational programs to encourage use of public toilets plus adequate prescription of ciprofloxacin might help control typhoid here in the future.

Conclusions

Although Ghana implemented several measures to control typhoid, our study found that, depending on the region, *S. enterica* serovar Typhi remains the most prevalent bacterial species causing BBSI. This finding is in agreement with a recent study from the Ashanti region, where 12.4% of BBSI were caused by *S. enterica* serovar Typhi (*14*). In addition, emergent ciprofloxacin resistance has been described in Accra, the capital of Ghana (*15*). Therefore, the implementation of bacteriologic diagnosis should be considered even in smaller hospitals in a rural African setting to monitor pathogen distribution and resistance rates.

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This article is dedicated to our friend, Nicholas Amgborme from Eikwe, who passed away much too soon.

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Isolation and Phylogenetic Grouping of Equine Encephalosis Virus in Israel

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During 2008–2009 in Israel, equine encephalosis virus (EEV) caused febrile outbreaks in horses. Phylogenetic analysis of segment 10 of the virus strains showed that they form a new cluster; analysis of segment 2 showed ≈92% sequence identity to EEV-3, the reference isolate. Thus, the source of this emerging EEV remains uncertain.

Equine encephalosis is an arthropod-borne, noncontagious, febrile disease of horses. It was first described >100 years ago by A. Theiler (*1*) under the name equine ephemeral fever. The disease is caused by *Equine encephalosis virus* (EEV; genus *Orbivirus*: subfamily *Sedoreovirinae*: family *Reoviridae)* (*2*,*3*), which is transmitted by *Culicoides* spp. biting midges (*4*). Before 2008, EEV had been isolated only in South Africa, where 7 antigenically distinct serotypes, EEV-1–7, have been identified and characterized (*3*).

Orbiviruses encode at least 7 structural and 4 nonstructural (NS) proteins from 10 linear dsRNA genome segments (*5*). The smallest genome segment, segment 10 (Seg-10), encodes NS3, which mediates the release of virus particles from infected cells, and NS3A. The second largest of the EEV genome segments, Seg-2, encodes virus protein (VP) 2, the larger of the 2 outer-capsid proteins. By analogy with bluetongue virus (BTV), the *Orbivirus* type species, the virus serotype is determined by the specificity of interactions between VP2 and neutralizing antibodies generated during infection of the mammalian host. Consequently, VP2 and Seg-2 show sequence variations that correlate with serotype and, thus, can be used to determine the virus serotype (*6*).

From October 2008 through January 2009, a febrile horse disease that was diagnosed as equine encephalosis was observed in dozens of stables across Israel (*7*). The recent emergence of novel orbivirus strains (including BTV and epizootic hemorrhagic disease virus) in Europe, North America, Asia, and Australia (*8*) is of major concern to the worldwide livestock industry. Furthermore, the similarity of EEV to African horse sickness virus, one of the most devastating pathogens of equids, warranted further investigation of the outbreaks and molecular characterization of the virus. The molecular and sequence analyses reported here confirm the existence of EEV in Israel and identify the virus and its serotype, as well as its phylogenetic roots.

The Study

During October–November 2009, samples of whole blood from 8 febrile horses (H1–8; temperatures 39.5°C–42°C) in Israel were collected into EDTA tubes and analyzed at the Koret School of Veterinary Medicine (Hebrew University, Rehovot, Israel). Vero cell (American Type Culture Collection, Manassas, VA, USA) culture results of blood from H3, H5, and H8 were positive for EEV (Table 1; Figure 1).

Total RNA was extracted from the fifth and sixth passages of all 3 samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) to obtain sufficient viral load for the subsequent analyses and replications. RNA was reverse transcribed into cDNA by using the Verso cDNA Kit (Thermo Fisher Scientific, Epsom, UK). PCR amplification of the gene encoding NS3 (Seg-10) was performed on the 3 isolates by using GoTaq Green Master Mix (Promega, Madison, WI, USA) with the following primers: 5′-^{1}GTT AAG TTT CTG CGC CAT GT^{23}-3′, 5′-^{741}GTA ACA CGT TTC CGC CAC G^{760}-3′. Thermal cycling conditions for the PCR were as previously described (*9*); the primer annealing temperature was modified to 53.5°C. PCR products were purified by using a cDNA purification kit (ExoSAP-IT; USB, Cleveland, OH, USA), and sequencing was conducted by BigDye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, USA) in an ABI 3700 DNA Analyzer (Applied Biosystems) by using ABI data collection and sequence analysis software. Further analysis of the NS3 sequence was performed with Sequencer software, version 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). Sequences were deposited in GenBank under accession nos. HQ441245 for H5, HQ441246 for H3, and HQ441247 for H8. The NS3 genes (Seg-10) were compared with those of different EEVs (*9*) and other related orbiviruses (Table 2). Phylogenic trees were generated by using the neighbor-joining and maximum-likelihood

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Table 1. Clinical signs for horses whose blood was tested to determine the cause of a febrile disease, Israel, October–November 2009

Horse no.	Clinical signs	Date of first clinical sign	Duration of clinical signs, d	Date of blood collection	Virus isolated*
1	Temperature 39.5°C, lack of appetite	Oct 25	5	Nov 2	No
2	Temperature 39.5°C, lack of appetite	Oct 29	3	Nov 2	No
3	Temperature 40°C, lack of appetite	Oct 30	3	Nov 3	Yes
4	Temperature 39.5°C, lack of appetite	Nov 8	Unknown	Nov 8	No
5	Fever, colic, lethargy, congested mucous membranes, rapid pulse, lack of appetite	Nov 5	5	Nov 9	Yes
6	Temperature 39.5°C, lack of appetite	Nov 17	4	Nov 22	No
7	Temperature 42°C, apathy	Nov 25	2	Nov 26	No
8	Temperature 39.7°C, lack of appetite, colic	Nov 26	3	Nov 27	Yes

*Positive cases were confirmed by reverse transcription PCR of dsRNA genome segment 10.

methods (Phylip Inference Package version 3.68, Seqboot Program; J. Felsenstein, University of Washington, Seattle, WA, USA) to create 100 datasets (bootstrapping) and the DNA Maximum Likelihood Program version 3.5 (http://cmgm.stanford.edu/phylip/dnaml.html) to construct the trees. Finally, the Consense program version 3.5c (http://cmgm.stanford.edu/phylip/consense.html) was used to create a final consensus tree for our dataset. Broadhaven virus, a tick-borne orbivirus, was used as the outgroup in the phylogram for the gene encoding NS3.

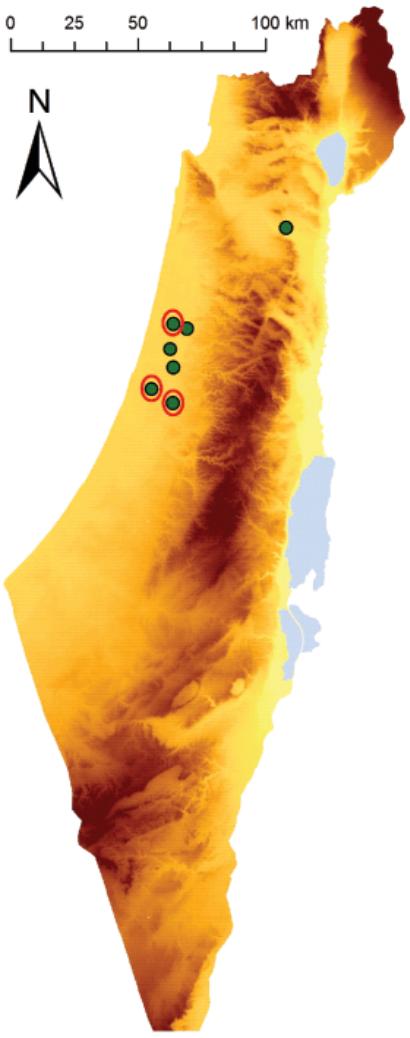

Figure 1. Geographic location of farms in Israel with horses showing signs of equine encephalosis virus (EEV) infection. Eight horses with suspected EEV infection lived on 7 farms. Red circles indicate farms with EEV-positive cases.

The phylogenetic analyses of EEV Seg-10 grouped the Israeli isolates with other EEV isolates but as a distinct group with no close relation to African horse sickness virus, BTV, or epizootic hemorrhagic disease virus. Within the EEV group, 3 discrete clusters (A, B, C) were recognized; the Israeli isolates formed one of these clusters (C; Figure 2). The Israeli isolates have 85%–86% nt identity to cluster A and 75%–76% nt identity to cluster B.

In addition, full-length cDNA copies of individual EEV (from H3 and H8) genome segments were synthesized and amplified by reverse transcription PCR by using the anchor spacer–ligation method as described (*10*,*11*). Partial sequences (for the upstream 450 bp) of Seg-2 from the different Israeli isolates were identical, showing 92.3% nt and 95.7% aa sequence identity with Seg-2 and VP2 of the Kaalplaas isolate, the reference isolate of EEV-3 (GenBank accession numbers are listed in Table 2). Previous phylogenetic comparisons of Seg-2/VP2 from different BTV types showed a maximum of 71% nt and 78% aa acid identity between serotypes (*6*), indicating that the isolates from Israel also belong to EEV type 3.

Conclusions

Equine encephalosis virus has long been enzootic to southern Africa, but it has not been isolated in other parts of the world. We report the characterization of an EEV strain isolated outside of Africa. Phylogenetic analysis of Seg-2 showed 92% sequence identity to EEV-3 (Kaalplaas).

Analysis of Seg-10 (the gene encoding NS3) of different orbiviruses showed 2 clusters of South African EEV strains (A and B), in agreement with previously published studies (*9*). These 2 clusters appear to correlate with the geographic origins of the viruses in South Africa,

Table 2. GenBank accession numbers for orbiviruses used for phylogenic analyses of strain isolated from horses in Israel, 2009*

Virus	Strain	Accession no. Seg-2	Accession no. Seg-10
EEV-1 (Cascara)	FLD1		AY115878
	FLD2		AY115876
	FLD3		AY115875
	FLD4		AY115877
	Ref		AY115865
	Ref†		AY115864
EEV-2 (Gamil)	Ref		AY115871
EEV-3 (Kaalplaas)	FLD1		AY115874
	Ref	HQ630933	AY115867
EEV-4 (Bryanston)	Ref		AY115868
EEV-5 (Kyalami)	Ref		AY115869
EEV-6 (Potchefstroom)	Ref		AY115866
	FLD1		AY115872
	FLD2		AY115873
EEV-7 (N Rand)	Ref		AY115870
AHSV-2			AF276700
AHSV-4			AJ007305
AHSV-7			AJ007306
BRDV			M83197
BTV- 2			AF135224
BTV-12			AF135227
PALV (Chuzan)			AB018091
EHDV-1			NC_013405.1
EHDV-2			AM745086.1
Israel EEV H5	Animal H5		HQ441245
Israel EEV H3	Animal H3 (ISR2009/20)‡	JF495411	HQ441246
Israel EEV H8	Animal H8 (ISR2009/21)‡	JF495412	HQ441247

*Seg, dsRNA genome segment; EEV, equine encephalosis virus; FLD, field strain; ref, reference strain; N Rand, North Rand; AHSV, African horse sickness virus; BRDV, Broadhaven virus; BTV, bluetongue virus; PALV, Palyam virus; EHDV, epizootic hemorrhagic disease virus; H5, H3, H8, horses 5, 3, 8.
†Unknown origin.
‡Isolate numbers for the *Orbivirus* reference collection (www.reoviridae.org/dsRNA_virus_proteins/ReoID/EEV-isolates.htm).

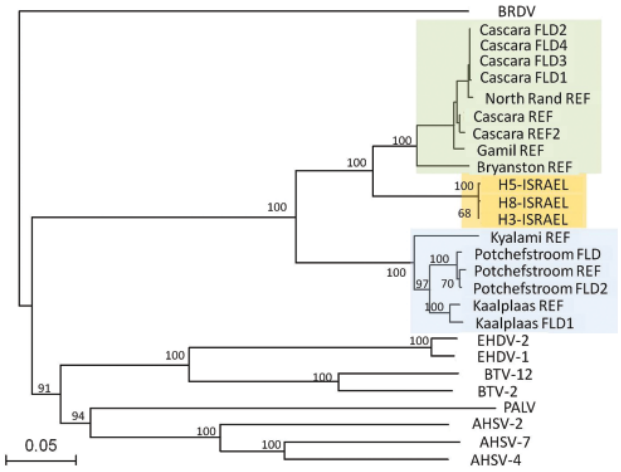

Figure 2. Phylogeny of equine encephalosis virus (EEV) segment 10 (nonstructural protein 3 gene) isolated from horses in Israel in 2009. The phylogenic tree was constructed by using the neighbor-joining method and bootstrapped with 100 replicates. Branch lengths are indicative of the genetic distances between sequences. Other orbiviruses were included for reference, with Broadhaven virus (BRDV) selected as the outgroup. The 3 suggested EEV clusters are marked green (cluster A), blue (cluster B), and orange (cluster C, representing the isolates from Israel). FLD, field strain; REF, reference; H3, H5, H8, horse 3, 5, and 8; EHDV, epizootic hemorrhagic disease virus; BTV, bluetongue virus; PALV, Palyam virus; AHSV, African horse sickness virus. Scale bar indicates nucleotide substitution per site.

independent of their isolation date. It has been suggested that the 2 EEV Seg-10 clusters in South Africa are related to the distribution of their *Culicoides* spp. midge vectors, *C. imicola* (*senso stricto*) and *C. bolitinos*. The former is the most abundant *Culicoides* spp. midge in Israel (*12*). However, the EEV isolates from Israel group as a distinct cluster (C) with similar distances to the 2 South African clusters, raising questions concerning the geographic origin of this virus. A similar finding has been observed in African horse sickness virus Seg-10, which also forms into 3 distinct groups (*13*).

The question of how and when the virus was initially introduced to Israel remains unanswered. Because the clinical manifestations of equine encephalosis are usually mild, it is often overlooked and underdiagnosed. EEV could have been introduced to Israel before the virus was first isolated in 2009. Alternatively, the virus might have been introduced into neighboring countries and transmitted into Israel by infected vectors carried by winds, as described for other orbiviruses (*14*,*15*). The fact that the Israeli strain of EEV-3 grouped in a different cluster than the 2 South African strains, supports the idea that it has evolved in this region for a sufficient time to accumulate these changes and most likely was not recently introduced into Israel from South Africa.

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Prevalence and Molecular Characterization of *Cyclospora cayetanensis*, Henan, China

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To determine prevalence of *Cyclospora cayetanensis* infection in Henan, China, we conducted a study of 11,554 hospital patients. Prevalence was 0.70% (95% confidence interval 0.70% ± 0.15%), with all age groups infected. Most cases were found in the summer. Minor sequence polymorphisms were observed in the 18S rRNA gene of 35 isolates characterized.

Cyclospora cayetanensis, a protozoan that causes watery diarrhea, fatigue, abdominal pain, weight loss, and inappetence, is endemic to some nonindustrialized countries (*1*–*4*). In industrialized countries, the infection has been traditionally associated with diarrheal illness in travelers to disease-endemic regions. However, since the 1990s, many foodborne and several waterborne outbreaks have been reported in North America (*2*,*3*).

Henan is an agricultural province in central China with a population of >100 million. To better understand the prevalence of cyclosporiasis and genetically characterize *C. cayetanensis*, we conducted a 23-month investigation of cyclosporiasis in patients treated at hospitals in the province.

The Study

The study was conducted in 2 urban areas, Zhengzhou and Kaifeng. A total of 11,554 (6,939 male; 4,615 female) child and adult patients at 3 hospitals (Huai River Hospital and 155th Liberation Army Hospital, Kaifeng, and Number One People's Hospital, Zhengzhou) were enrolled in this study during June 2007–December 2008 and July–October 2009. Only data concerning age, sex, and diarrhea presence or absence were made available to laboratorians. One stool specimen from each patient was examined for *Cyclospora* spp. by microscopy of fecal materials that were concentrated by the formalin-ethyl acetate sedimentation method and stained with the modified acid-fast staining technique (*1*). We used the χ^2 test to compare the frequency of *Cyclospora* spp. infection among patients according to age group and sex and by season of the year. Differences were considered significant if $p<0.05$.

Cyclospora oocysts were detected in 81 (0.70%; 95% confidence interval [CI] 0.70% ± 0.15%) of 11,554 patients by microscopy (Table 1). Oocysts were variably stained from light pink to deep purple or remained unstained (Figure 1, panel A). They measured 8.61 ± 0.32 × 8.64 ± 0.33 μm, with a length/width shape index of 1.01 (n = 55; Figure 1, panel B), and showed typical blue autofluorescence under an epifluorescence microscope with a 330–380 nm excitation filter (Figure 1, panel C). Oocysts sporulated at 32°C ≤13 days in 2.5% potassium dichromate.

Cyclospora oocysts were seen in samples from patients in all age groups, although the age group 7–17 years had the highest detection rate (1.47%, 95% CI ± 0.91%; Pearson correlation >0.05) (Table 1). No significant difference was found in detection rate by patient sex; the infection rates for female and male patients were 0.74% (34/4,615, 95% CI ± 0.25%) and 0.68% (47/6,939, 95% CI ± 0.19%), respectively (Pearson correlation >0.05). The overall infection rate of *C. cayetanensis* was similar between Zhengzhou and Kaifeng: 0.60% (95% CI 0.60 ± 0.19%) versus 0.79% (95% CI ± 0.22%) (Pearson correlation >0.05; Table 1).

The prevalence of cyclosporiasis was markedly seasonal, occurring only during July through November, with a sharp peak in August (Table 2). The occurrence of cyclosporiasis coincided with the rainy season and lagged slightly behind the peaks for mean temperature and precipitation in the year (Figure 2).

Among patients in this investigation, 5,533 had records documenting presence or absence of diarrhea at the time of specimen submission. The detection rate of *Cyclospora* oocysts was significantly higher for patients with diarrhea (2.97% or 12/404; 95% CI 2.97 ± 0.52%) than for patients without diarrhea (0.66% or 34/5,129; 95% CI 0.66 ± 0.22%) (Pearson correlation <0.01).

Genomic DNA was extracted from *Cyclospora* oocysts from 35 randomly chosen patients; the oocysts were purified by sucrose gradient centrifugation by using the Mag Extractor-Genome kit (Toyobo Co. Ltd,

College of Animal Science and Veterinary Medicine-Henan Agricultural University, Zhengzhou, People's Republic of China (Y. Zhou, B. Lv, Q. Wang, R. Wang, F. Jian, L. Zhang, C. Ning, M. Qi, H. Yao, J. Zhao, X. Zhang, Y. Sun, K. Shi); Henan University Huaihe Hospital, Kaifeng, People's Republic of China (K. Fu, Y. Wang); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (M.J. Arrowood, L. Xiao)

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Table 1. Prevalence and distribution of *Cyclospora cayetanensis* by patient age, sex, and residential area, Henan Province, China, 2009–2010*

Variable	No. infected/ no. patients	Detection rate (95% CI), %
Sex†		
M	47/6,939	0.68 (± 0.01)
F	34/4,615	0.74 (± 0.25)
Age, y		
≤6	6/926	0.65 (± 0.52)
7–17	10/678	1.47 (± 0.91)
18–28	13/1,301	1.00 (± 0.17)
29–44	15/2,343	0.64 (± 0.32)
≥45	37/6,306	0.59 (± 0.19)
Area†		
Kaifeng	48/6,093	0.79 (± 0.22)
Zhengzhou	33/5,461	0.60 (± 0.20)
Total	81/11,554	0.70 (± 0.15)

*CI, confidence interval.
†Pearson correlation >0.05.

Osaka, Japan). A nested PCR was used to amplify a 501-bp fragment of the 18S rRNA gene (*5*). All 35 microscopy-positive specimens produced the expected PCR product and were sequenced successfully. The *C. cayetanensis* identity was established by comparing the sequences obtained with a full sequence (AF111183) of the 18S rRNA gene of *C. cayetanensis* from Guatemala and 3 partial sequences (AB368541, AB368542, and AB368543) from Japan. In addition, this comparison revealed the presence of 2 polymorphic sites at nucleotide positions 687 and 696 of the full gene, with a few other inconsistent nucleotide substitutions at other positions. Thus, 3 isolates had a C to T substitution at position 687, and 5 isolates plus AB368542 and AB368543 had a C to T substitution at position 696. Nevertheless, similarities among the 35 *C. cayetanensis* isolates and reference sequences were 99.6%–100% at the 18S rRNA locus. Representative sequences of the partial 18S rRNA gene generated were deposited in the GenBank database under accession nos. GQ292774–GQ292782, FJ009120–FJ009129, and EU860998–EU861002.

Conclusions

The overall infection rate of 0.70% (95% CI ± 0.15%) in this 23-month investigation in 2 Henan Province cities is similar to data previously obtained in an urban area in the neighboring Anhui Province (0.92%, 95% CI ± 1.04%) (*6*) and studies in Albania (0.63%, 95% CI ± 0.55%) (*7*) and Tanzania (0.91, 95% CI ± 1.25%) (*8*) but higher than the infection rate in the United Kingdom (0.07%, 95% CI ± 0.07%) (*9*). The rate is significantly lower than those in surveys conducted in healthy populations in other countries (*3*,*10*). The fact that *C. cayetanensis* does not always cause clinical symptoms might have contributed to the differences in infection rates among studies (*3*). In addition, *C. cayetanensis* is mainly spread by consumption of contaminated fresh produce and water (*2*,*3*). In China, persons are less likely to eat raw vegetables and drink unboiled tap water, which are known sources of sporulated *C. cayetanensis* oocysts in nonindustrialized countries (*3*). As expected, in this study the *C. cayetanensis* detection rate was higher for patients with diarrhea than in those without diarrhea because cyclosporiasis has been associated with mild-to-moderate self-limiting diarrhea in children and protracted and severe diarrhea in HIV-positive adults (*2*,*3*,*11*).

In addition to differences in study populations, socioeconomic conditions, and cultural habits, local climatic factors may have contributed to the variation in prevalence of cyclosporiasis observed in different studies. In this study, transmission of *C. cayetanensis* was seasonal; of the 81 cases of cyclosporiasis detected, 95% (95% CI 95% ± 4.7%) occurred during July 1–September 30, the hottest and rainiest months of the year. This result differs from the peak transmission of *C. cayetanensis*

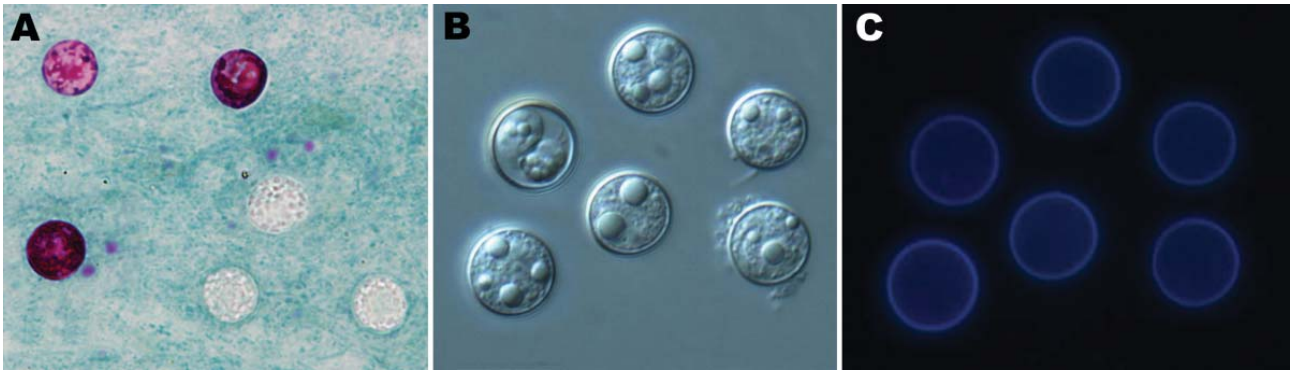

Figure 1. *Cyclospora cayetanensis* oocysts under light microscopy of stool smears stained with the modified acid-fast stain (A), showing differential interference contrast microscopy of wet mount (B), and results of epifluorescence microscopy using a 330–380 nm ultraviolet excitation filter (C). Two oocysts are stained at different intensities (A), and a partially sporulated oocyst is seen (B). Original magnifications ×1,000.

Table 2. Monthly prevalence of *Cyclospora cayetanensis* in Kaifeng and Zhengzhou, Henan Province, China, 2009–2010*

Month	No. infected/ no. patients	Infection rate (95% CI), %
Jan	0/123	0
Feb	0/89	0
Mar	0/595	0
Apr	0/905	0
May	0/388	0
Jun	0/410	0
Jul†	21/1,1814	1.16 (± 0.49)
Aug‡	44/2,529	1.74 (± 0.51)
Sep†‡	12/1,860	0.65 (± 0.37)
Oct†‡	3/1,638	0.18 (± 0.21)
Nov†‡	1/740	0.14 (± 0.27)
Dec	0/463	0

*CI, confidence interval.
†Pearson correlation <0.05.
‡Pearson correlation <0.01.

in some other geographic areas. For example, in Lima, Peru, *C. cayetanensis* prevalence peaks in warm months (December–May) in the absence of rain (*1*). In contrast, in Haiti where ambient temperature is high year-round, *C. cayetanensis* infection coincides with the cooler part of the year (*10*).

Thus far, 19 species of *Cyclospora* spp. have been described (*3*), but only 4 of them, *C. cayetanensis* and 3 species from nonhuman primates, have been characterized by sequence analysis of the 18S, 5.8S, and 28S rRNA genes and the associated internal transcribed spacer (*3*,*12*). Currently, no reliable genotyping or subtyping tools are available for the investigation of *C. cayetanensis* transmission, the only known *Cyclospora* species that infects humans (*3*). In our study, detection of *C. cayetanensis* in human stool specimens was confirmed by DNA sequencing of the partial 18S rRNA gene. We identified 2 polymorphic sites in the partial 18S rRNA gene of *C. cayetanensis*, although the meaning of the sequence polymorphism remains unclear.

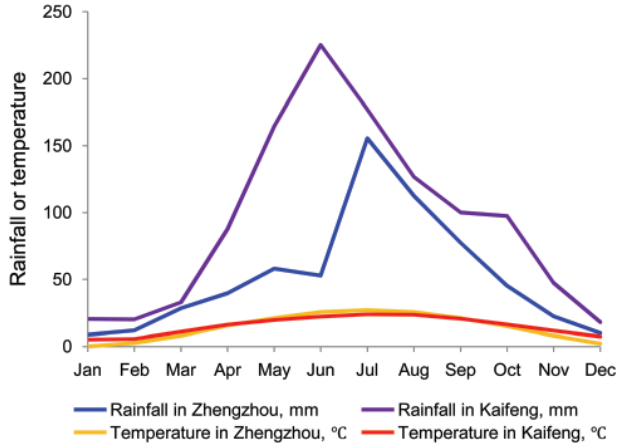

Figure 2. Mean monthly rainfall and mean daily average temperature recorded for Zhengzhou and Kaifeng, Henan Province, China, 1995–2008. Data source: www.chinaweatherguide.com.

In conclusion, *C. cayetanensis* infects humans in Henan Province at a relatively low frequency but with a marked seasonality. Additional research is needed to determine disease effects, transmission routes, and risk factors for *C. cayetanensis* infection in humans in Henan and elsewhere in China. Research could be facilitated by development of genotyping and subtyping tools for the differentiation and tracking of *C. cayetanensis* isolates.

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Yellow Fever Virus Vaccine–associated Deaths in Young Women[1]

Stephen J. Seligman

Yellow fever vaccine–associated viscerotropic disease is a rare sequela of live-attenuated virus vaccine. Elderly persons and persons who have had thymectomies have increased susceptibility. A review of published and other data suggested a higher than expected number of deaths from yellow fever vaccine–associated viscerotropic disease among women 19–34 years of age without known immunodeficiency.

Yellow fever virus (YFV) vaccine had been considered the safest of the live-virus vaccines. Rare neurologic adverse events, called yellow fever vaccine–associated neurotropic disease (YEL-AND), have long been recognized but are seldom fatal. However, in 2001, the vaccine was found to cause a serious, frequently fatal, multisystemic illness, called yellow fever vaccine–associated viscerotropic disease (YEL-AVD), which resembles the illness it was designed to prevent (*1*–*3*). According to reports from the Vaccine Adverse Event Reporting System (VAERS) (www.vaers.hhs.gov), the frequency of YEL-AVD in US vaccinees was 0.4 per 100,000 doses of vaccine administered (*4*).

Elderly persons (*4*) and patients who have undergone thymectomies secondary to thymoma (*5*) are recognized as groups at risk for YEL-AVD. However, several case reports of YEL-AVD in young women raise concern that women of childbearing age might also be at increased risk (*6*–*10*).

The Study

To investigate the possibility of age- and sex-specific risk groups, a comprehensive YEL-AVD dataset (Table 36-30 in *11*), was analyzed (Figure). This dataset has the advantage of having been compiled with information that is not otherwise publicly available: data from the Centers for Disease Control and Prevention (Atlanta, GA, USA), patient charts, and vaccine manufacturers (T.P. Monath, pers. comm.).

Two concentrations of cases were evident: cases in men ≥60 years of age who survived and in women 19–34 years of age who died. Although selection bias may have influenced the cases reported, the sex-specific survival rates for these 2 age groups statistically differed: 21% (3/14) versus 0% (0/6) ($p = 0.002$ by Fisher exact test). In addition to the surprisingly low case-fatality rate for elderly men, only 2 of the 4 patients who had undergone thymectomy and had YEL-AVD died.

Searches for additional YEL-AVD cases among women of childbearing age (15–44 years) and of comparably aged men included review of published cases through PubMed (www.ncbi.nlm.nih.gov/sites/entrez) and reports from the ProMED Web site (http://apex.oracle.com/pls/otn/f?p=2400:1000:). In follow-up of a ProMED listing, 1 case was supplied by Bio-Manguinhos (Rio de Janeiro, Brazil), a producer of YFV vaccine. VAERS also was searched. Information was sought from authors of case reports. Cases listed in VAERS were excluded if another explanation for the adverse event was evident in the case description or if they contained insufficient information to classify the event as YEL-AVD.

A total of 9 fatal cases of YEL-AVD in young adults, all women, were found (Table). Six cases were included in the report by Monath et al. (*11*), and 3 cases were found through the author's search. The eldest of the 9 case-patients was 34 years of age. One case listed by Monath et al. occurred in 1975 and was originally thought to be yellow fever but was documented as vaccine-related ≈2 decades later (*13*). This patient's age is not known, but she was reported to be a young woman (P. Vasconcelos, pers. comm.).

Three fatal cases of possible YEL-AVD among young women reported in VAERS were excluded from the Table because information was insufficient to document the diagnosis. Two other cases of suspected YEL-AVD, 1 each in an 18-year-old man and a 24-year-old woman, occurred outside the United States. Hence, these patients could have come from regions where yellow fever was endemic and thus might have had wild-type yellow fever.

Also excluded from the Table are 2 cases reported in the published literature: 1 in a 23-year-old woman with a partial C4 deficiency and discoid lupus erythematosis hospitalized with severe YEL-AND and YEL-AVD who survived (*14*) and 1 in a 43-year-old woman with systemic lupus erythematosus who died (*10*). The first was excluded because she survived, had clinical features that included YEL-AND, and had known immunodeficiency. For the second patient, a history of disseminated lupus

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[1]Data previously presented at a National Institutes of Health–supported Northeast Biodefense Center conference, New Paltz, New York, USA, November 2, 2010.

Figure. Cases of yellow fever vaccine–associated viscerotropic disease, by patient age, sex, and outcome. One woman who died and whose precise age is unknown was a young woman (P. Vasconcelos, pers. comm.) arbitrarily depicted as being 23 years of age. Data obtained from Table 36-30 in (*11*).

erythematosis, the relatively long interval (30 days) until death (in contrast to the 9–14 days in the other women), and her older age suggest that her susceptibility to the vaccine differed from those listed in the Table.

In several investigations of YEL-AVD cases, extensive sequence analyses did not indicate any substantial evidence of reversion of the vaccine to virulence (*1*,*10*). Two varieties of YFV vaccine are available: the 17DD vaccine produced in Brazil and used in South America and the 17D-204 vaccine (YF-Vax, Sanofi Pasteur, Swiftwater, PA, USA; and Stamaril, Sanofi Pasteur, Lyon, France) used elsewhere. Six cases listed in the Table occurred in 17DD vaccine recipients in South America, and 3 occurred in persons who received 17D-204 as prospective travelers.

The limited racial information available indicates that cases were not confined to persons of any particular racial group. Of 3 case-patients for whom racial information was available, 1 each was described as Caucasian (*8*), black (*2*), and of Pacific Islander ancestry (*7*).

Table. Characteristics of fatal yellow fever vaccine–associated viscerotropic disease in women of childbearing age who had no known immunologic defects*

Age, y	Country	Year	Vaccine	Days after vaccination		Possible predisposing factors	Virus detection	Neutralizing antibody test results	Reference
				Onset	Death				
19	Brazil	2001	17DD†	2	10	None known	+ RT-PCR liver and spleen		(*12*)
22	United States	2002	17D-204 YF-Vax‡	2	10	None known	YF viral antigen in multiple organs, i.e., liver, lungs, brain, heart, spleen, kidney, lymph nodes		(*7*)
22	United States	2005	17D-204 YF-Vax‡	2	11	See text for postmortem description of thymus	Plasma virus 1.1 × 10^5 PFU/mL	2,560, day 10	(*9*)
22	Brazil	2000	17DD†	4	11	Hepatitis A and nephritis as a child	+ Culture	IgM +	(*2*)
23	Peru	2007	17DD†	1	9	Acne rosacea	Viral RNA lung 7.6 × 10^6 and serum 3.9 × 10^6 PFU equivalents/mL	160 (by PRNT), day 9	(*10*)
Young adult §	Brazil	1975	17DD†	5	9	None known	+ Culture		Table 36-30 in (*11*), (*13*)
24	Peru	2007	17DD†	<1	14	Egg allergy	Viral RNA liver 1.1 × 10^4 and brain 4.2 × 10^3 PFU equivalents/mL	10,240 (by PRNT), day 11	(*10*)
26	Spain	2004	17D-204¶	4	10	None known	+ Culture liver, kidney, plasma; real-time PCR liver 6.2 × 10^9 genome equivalents/g	512 (by microneutralization assay), day 8	(*8*)
34	Brazil	2009	17DD†	1	11	None known	RT-PCR + d 10		#

*+, positive test result; RT-PCR, reverse transcription PCR; YF, yellow fever; Ig, immunoglobulin; PRNT, plaque-reduction neutralization test. Blank cells indicate information is not in the reference cited.
†Bio-Manguinhos, Rio de Janeiro, Brazil.
‡Sanofi Pasteur, Swiftwater, PA, USA.
§The age of this patient is not known, but she was a young woman (P. Vasconcelos, pers. comm.).
¶Stamaril, Sanofi Pasteur, Lyon, France.
#R. Menezes-Martins, pers. comm.

Despite the known association of thymectomy with YEL-AVD, the only observation on possible thymic disease in the reports of the 9 cases is the statement that, at autopsy of a 22-year-old woman from the United States, the thymus was replaced by fat (Table 36-30 in *11*). However, the accuracy of the finding should be considered in the context that, at surgery, experienced cardiothoracic surgeons may have difficulty in distinguishing thymus from adipose tissue (R.L. Berger, pers. comm.) and that the thymus was not examined histologically (R.V. Ridenour, III, pers. comm.). Because the thymus may be difficult to separate from surrounding adipose tissue and is infrequently a source of disease, pathologists, at least in the United States, do not routinely examine it histologically at autopsy (I. Argani, pers. comm.). Thymic deficiencies such as Sutton thymic dysplasia (fatal viral infection in young women with a dysplastic thymus) (*15*) have yet to be excluded.

Conclusions

Although accurate denominators are not available for calculating age- and sex-specific incidence of YEL-AVD, the number of fatal YEL-AVD cases among women of childbearing age appears to be higher than expected. Further investigation should include ascertainment of family history; exploration of contraceptive medications or occult pregnancy as possible predisposing factors; examination of the thymus at postmortem, including thymus weight and histology; further evaluation of possible complement defects; and evaluation of any associations with autoimmune disease.

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Unexpected Rift Valley Fever Outbreak, Northern Mauritania

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During September–October 2010, an unprecedented outbreak of Rift Valley fever was reported in the northern Sahelian region of Mauritania after exceptionally heavy rainfall. Camels probably played a central role in the local amplification of the virus. We describe the main clinical signs (hemorrhagic fever, icterus, and nervous symptoms) observed during the outbreak.

From late September through the beginning of October 2010, unprecedented rainfall created large ponds in the oases of the Saharan region of Adrar, northern Mauritania (Figure 1). Such rains had not been observed for decades; the local residents refer to 1956 (locally known as the "year of the fever") to describe similar events. This climatic event translated into unusual growth of vegetation, attracting shepherds and pastoralists from remote areas, including the southern and southeastern regions of the country. It also favored high densities of mosquitoes, mainly from the genus *Culex* and *Anopheles* (*Cx. quinquefasciatus, An. pharoensis, An. protoriensis, Cx. poicilipes, An. gambiae, Aedes vexans, Cx. antenatus, An. rufipes, Mansonia uniformis, An. ziemani*); some of these species were known to be competent vector species for major arboviruses.

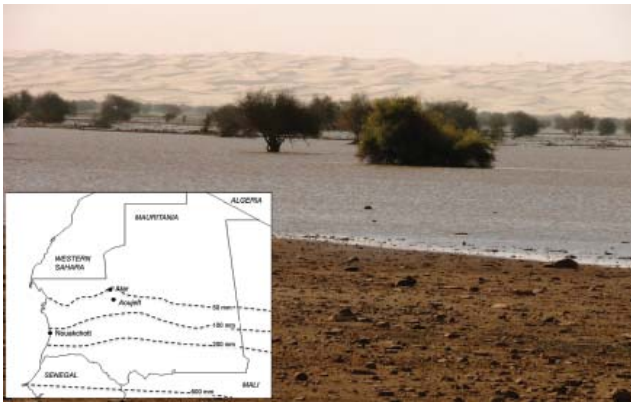

Figure 1. Lefrass Oasis, 30 km north of Atar, one of the main outbreak foci of an outbreak of Rift Valley fever in camels, northern Mauritania. Inset shows the location of Atar and Aoujeft and the isohyets (average during 1965–2002; source: Food and Agricultural Organization of the United Nations, Land and Water Development Division).

A few weeks after these rains, severe outbreaks of malaria and Rift Valley fever (RVF) were reported in several oases (*graret*) of the Adrar region. Notably, the first probable reportable case in livestock was in a sick dromedary camel and occurred during the last week of October 2010 in the Aoujeft area; the camel's signs were similar to those of pasteurellosis. The herdsman slaughtered the animal before it died but delayed the cutting up of the meat because of the remote location. Subsequently, the uncooked meat was shared within the extended family, and within a few days, several people died with intestinal and hemorrhagic symptoms. Health authorities requested testing for several pathogens, including Crimean-Congo hemorrhagic fever and RVF; results were positive for RVF. Although these persons likely did not become infected through the consumption of meat (the fall in pH during meat maturation rapidly destroys the virus) (*1*), the virus was obviously circulating intensively in this area at that time.

Two weeks after the index case, additional cases in camels, abortion storms in small ruminants, and human deaths (hemorrhagic fever, icterus, and nervous symptoms) were reported on a massive scale. At the end of December 2010, a total of 63 cases among humans, including 13 deaths, had been officially reported, but the true number is probably much higher due to the remoteness of the affected area. Of 14 initial blood samples from camels, 7 had positive test results by real-time reverse transcription PCR; the virus was isolated from 4 of those positive samples in the Laboratoire National d'Elevage et de Recherches Vétérinaires, Dakar, Senegal. The first serologic results obtained by the central

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Table. Serologic data obtained from different regions, overall and per host species during Rift Valley Fever outbreak, northern Mauritania, September–October 2010*

Region	All samples, no. (%)			Small ruminant samples, no. (%)			Camel samples, no. (%)		
	All samples	IgM/IgG positive	IgM positive†	All samples	IgM/IgG positive	IgM positive†	All samples	IgM/IgG positive	IgM positive†
Adrar	179	83 (46)	81 (45)	168	79 (47)	77 (46)	11	4 (36)	4 (36)
Brakna	17‡	3 (18)	0 (0)	2	0	0	10	3 (30)	0
Gorgol	8	2 (25)	2 (25)	8	2 (25)	2 (25)	0	0	0
Inchiri	57	32 (56)	31 (54)	57	32 (56)	31 (54)	0	0	0
Nouakchott	239	65 (27)	1 (0)	27	1 (4)	1 (4)	212	64 (30)	0
Nouadibou	46	20 (43)	8 (17)	0	0 (0)	0 (0)	46	20 (43)	8 (17)
Total	546	205 (37)	123 (23)	262	114 (43)	111	279	91 (33)	12 (4)

*Ig, immunoglobulin.
†IgM-positive samples within the IgM/IgG-positive population.
‡Includes 5 cattle samples; all had negative test results.

veterinary laboratory using a competitive ELISA (ID Screen Rift Valley Fever Competition Multispecies ELISA, ID-Vet, Montpellier, France) indicated an immunoglobulin (Ig) M/IgG prevalence of 33% in camels and 44% in small ruminants, respectively. IgM titers (*2*) were as high as 45% in Adrar and even reached 54% in the eastern Inchiri area 2 weeks after the index case in the camel was observed (Table).

Serologic evidence of RVF in camels is frequently reported (*3*), yet the description of clinical signs is rare (*4*). Some authors mention subclinical or mild forms (*5*) or even the capacity to carry the virus without clinical signs (*1*). In contrast, in the past, widespread abortion waves in camels were observed during RVF outbreaks in Kenya and Egypt and were associated with positive serologic test results (*6*,*7*). Furthermore, camels are suspected of playing a major role in the spread of RVF from northern Sudan to southern Egypt in 1977 (*8*). It should be noted that RVF virus was previously isolated from blood samples from healthy, naturally infected camels in Egypt and Sudan (*9*,*10*) and that experimental infections with RVF virus have induced no clinical signs in nonpregnant dromedaries (*3*).

During this outbreak, 2 clinical forms were observed in camels: a hyperacute form, with sudden death in <24 hours; and an acute form with fever, ataxia, ballooning, edema at the base of the neck, audible expiratory wheeze and ventral positional dyspnea, blood-tinged nasal discharge, icterus, severe conjunctivitis with ocular discharge and blindness, hemorrhages of gums and tongue, foot lesions, nervous symptoms, and abortions (Figure 2). When hemorrhagic signs developed, death usually occurred within a few days.

The current understanding of the outbreak is that the exceptional rainfall during September–October 2010 created highly favorable conditions for colonization and subsequent multiplication of competent vectors in these *grarets*. The virus was probably introduced rapidly through viremic animals transported by truck for grazing opportunities from various areas, including the south and southeastern regions of Mauritania where RVF is endemic

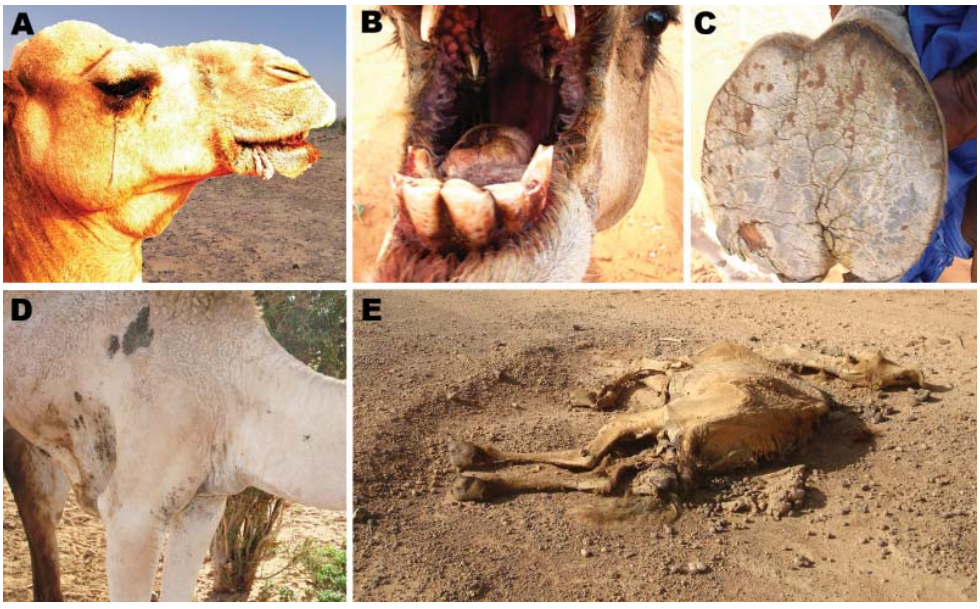

Figure 2. Observed clinical symptoms of Rift Valley fever in camels during field investigation in the Adrar region, northern Mauritania. A) Conjunctivitis and ocular discharge, hemorrhages of the gums, and edema of the trough; B) hemorrhages of gums and tongue; C) foot lesions (cracks in the sole) with secondary myasis; D) edema at the base of the neck; E) dead camel with sign of abortion, convulsions, and arching of the neck.

(*11*,*12*). To cope with this outbreak, veterinary and public health authorities took appropriate control measures, including restriction of livestock movement, re-allocation of locust control teams for mass insecticide spraying, and risk communication and public awareness campaigns aimed at the population at risk.

Conclusions

We report the unusual outbreak of RVF at a northern latitude and in an extremely arid region (although RVF has been reported in Egypt, where the Nile River helps spread the disease from the south). The high mortality rates and severe clinical signs observed among dromedary camels indicate that these animals played a major role in the epidemiology of this outbreak. The capacity of RVF-infected *Aedes* spp. eggs to survive in such an environment also needs further assessment. Indeed, increasing capacities for long-distance transportation, associated with increasing frequencies of extreme and hard-to-predict weather events, may create a challenging scenario for exotic diseases in general, and RVF in particular, to spread.

During the course of the outbreak in Adrar, the price of livestock decreased by 40%, which created an attractive opportunity for traders who potentially could further disseminate the virus. Also, the possible role of oases as relay points has for some time been seen as a major risk for the introduction of RVF in the Maghreb, where eco-climatic and entomologic conditions are favorable for its emergence. These possible risk factors and the unusual appearance of RVF in an arid region call for further strengthening of surveillance and sanitary capacities and policies.

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Seroconversion to Pandemic (H1N1) 2009 Virus and Cross-Reactive Immunity to Other Swine Influenza Viruses

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To assess herd immunity to swine influenza viruses, we determined antibodies in 28 paired serum samples from participants in a prospective serologic cohort study in Hong Kong who had seroconverted to pandemic (H1N1) 2009 virus. Results indicated that infection with pandemic (H1N1) 2009 broadens cross-reactive immunity to other recent subtype H1 swine viruses.

Pandemic (H1N1) 2009 was able to spread globally because it was antigenically divergent from contemporary human seasonal subtype H1N1 influenza viruses (*1*). Because we now recognize that pandemics can arise from influenza subtypes endemic in humans, it is essential that subtypes, H1 and H3 swine viruses be considered potential future pandemic candidates, together with other avian virus subtypes such as H2, H5, or H9. Thus, it becomes imperative to investigate herd immunity in humans to swine and avian influenza viruses of subtypes H1 and H3.

Influenza virus subtypes H1 and H3 of diverse lineages are endemic in swine and are globally widespread. Eurasian avian-like swine H1 viruses are found in Europe; triple reassortant swine subtypes H1 and H3 viruses remain entrenched in North America (*2*). In China, we have demonstrated the co-circulation of these lineages together with classical swine (CS) subtype H1 viruses and also documented the emergence of antigenically variant reassortant viruses with gene segments of ≥2 of these lineages. We previously showed a lack of herd immunity in humans to some of these swine virus lineages in serum samples collected before the 2009 pandemic (*3*). However, the spread of pandemic (H1N1) 2009 worldwide may generate cross-reactive herd immunity to some of these swine virus lineages, making them less likely candidates for future pandemics. In this study, we assessed the relationship between seroconversion to pandemic (H1N1) 2009 and cross-reactive antibody responses to other major subtype H1 swine viruses in humans.

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The Study

Twenty-eight paired serum samples from a prospective serologic cohort study in Hong Kong, in which participants seroconverted to the pandemic (H1N1) 2009 virus, were selected to represent persons of diverse ages (median 39.5 years, range 8–74 years). Details of the serologic cohort have been reported elsewhere (*4*). The first (prepandemic or baseline) serum sample from each person was collected during July–August 2009, and the second (postpandemic or convalescent-phase) serum sample was collected during November 2009–February 2010. The peak of the first pandemic wave in Hong Kong occurred in September 2009. Subtype H1 swine influenza viruses, representative of CS, Eurasian avian-like swine (EA), triple reassortant swine (TRIG), pandemic (H1N1) 2009 viruses, and selected reassortants between these lineages with diverse antigenic profiles, were selected from our surveillance of swine influenza viruses in China (*3*). Relevant viruses from other geographic regions were also included. Each pair of baseline and convalescent-phase serum samples was tested for antibodies by microneutralization tests using each swine influenza virus. The profile of serologic responses to these swine viruses is shown in the Figure.

In accordance with our selection criteria, all 28 persons seroconverted (rise in antibody titer from <20 to ≥40) to the pandemic (H1N1) 2009 virus; follow-up antibody titers ranged from 40 to 320 (Figure). As expected, no serologic response occurred to seasonal influenza (H1N1) virus A/HK/400599/2008. Because the postpandemic serum samples were collected 2–5 months after the peak of the pandemic, waning of antibody titers over a few months is expected to be relatively modest (*5*). Notably, although few of the prepandemic serum samples tested had evidence of antibody titers ≥40 to any of the swine H1 viruses, the convalescent-phase serum sample of most persons had detectable antibody titers to other influenza viruses: CS (H1N1) Sw4167 (93% seropositive at a titer of ≥40; geometric mean titer [GMT] 121.9) and Sw1304 (86%; GMT 107.7); TRIG Sw1110 (75%; GMT 50); EA SwNS29 (46%; GMT 40), and an antigenically variant EA virus from Hong Kong that had acquired a nonstructural gene segment from TRIG virus by reassortment (57%; GMT 53) (Table).

Because Hong Kong is a heavily urbanized environment, exposure of the study volunteers to live

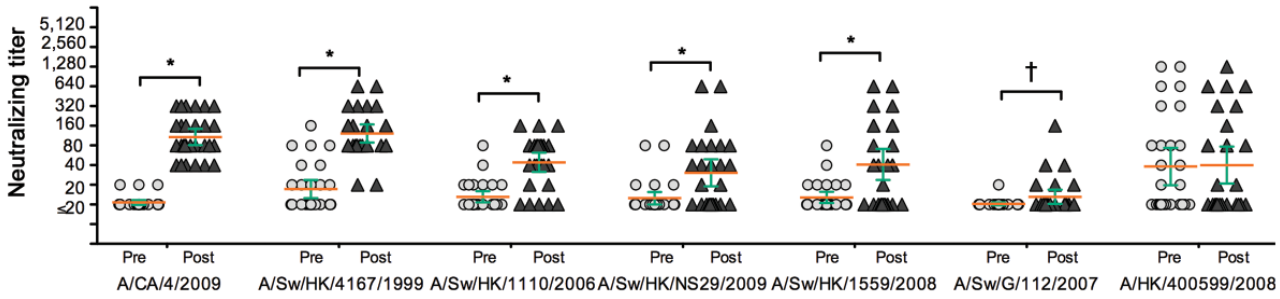

Figure. Neutralizing antibody titers to subtype H1 swine influenza viruses of the classical swine, North American triple reassortant, and Eurasian avian-like swine lineages in baseline (prepandemic [pre]) and convalescent-phase (postpandemic [post]) serum samples from 28 persons who seroconverted to pandemic (H1N1) 2009 infection, Hong Kong. Complete details on the serologic study cohort from which this subset is drawn are from (*4*). The pandemic A/California/4/2009 (H1N1) and seasonal influenza A/HK/400599/2008 (H1N1) viruses were used as controls. Orange lines indicate geometric mean titer; green error bars indicate 95% confidence intervals. *p≤0.05; †p≤0.01.

pigs during the 7-month study period is unlikely. Thus, the seroconversion to swine influenza viruses observed is not likely to be caused by infection with other swine influenza viruses. Notably, only 11% of these persons had neutralizing antibody titers to the EA virus SwG112, isolated in Ghent, Belgium. The difference between the EA viruses in Asia and Europe in this regard is worthy of further study. Because the hemagglutinin of EA virus is of avian origin, it is expected to cross-react poorly with subtype H1 of human or CS (derived from the 1918 pandemic H1 virus) derivation. What was unexpected was the observation that EA viruses isolated in China appear to manifest greater serologic cross-reactivity with pandemic (H1N1) 2009. Notably, little cross-reactivity occurred to 2 avian subtype H1 viruses isolated from wild birds in Hong Kong (data not shown).

A Poisson regression model of age as an indication of exposure for titer was used to look for evidence of age effects in both baseline and follow-up serum samples. The raw titers t (from the scale [<20, 20, 40, 80, …]) were transformed to outcome variable x (from the scale [0, 1, 2, 3, …]) in the following way: first, values of <20 were assigned the value of 10. Second, titers were divided by 10 and the logarithm taken (base 2). We used an uncorrected 95% statistical significance to test for preliminary evidence of an age effect. In the prepandemic serum samples, increasing age was significantly associated with increased antibody titers for pandemic (H1N1) 2009 (0.087; 95% confidence interval [CI] 0.002–0.720) and for TRIG virus 1110 (0.036; 95% CI 0.0009–0.062). Conversely, a significant negative relation with age for seasonal subtype H1N1 virus was found (–0.039; 95% CI –0.057 to –0.022). No significant age effects were found for other viruses. This age effect was lost in postpandemic infection serum samples, with the exception of antibody titers to the seasonal subtype H1N1 virus, which still had a negative association with age.

Table. Seroprevalence and GMT for swine influenza viruses of H1 subtype in prepandemic and postpandemic serum specimens from 28 persons who seroconverted for pandemic (H1N1) 2009, Hong Kong*

Virus	Virus abbreviation	Virus lineage (abbreviation)	No. (%) seroconverters†		GMT	
			Prepandemic	Postpandemic	Prepandemic	Postpandemic
A/California/4/2009 (H1N1)	Cal4	Pandemic (Pdm)	0	28 (100)	10.77	107.7
A/Swine/HK/4167/1999 (H1N1)	Sw4167	Classical swine (CS)	6 (21)	26 (93)	17.24	121.9
A/Swine/HK/1304/2003 (H1N?)	Sw1304	Classical swine reassortant (CSr)	7 (25)	24 (86)	16.41	105
A/Swine/HK/1110/2006 (H1N2)	Sw1110	Triple reassortant (TRIG)	2 (7)	21 (75)	13.13	44.16
A/Swine/HK/NS29/2009 (H1N1)	SwNS29	Eurasian avian-like (EA)	2 (7)	13 (46)	12.50	30.46
A/Swine/HK/1559/2008 (H1N1)	Sw1559	Eurasian avian-like reassortant (EAr)	2 (7)	16 (57)	12.81	41.00
A/Swine/G/112/2007 (H1N1)	Sw112	Eurasian avian-like (EA)	0 (0)	3 (11)	10.25	13.13
A/Swine/HK/201/2010 (H1N1)	Sw201	TRIG reassortant (TRIGr)	3 (11)	21 (75)	12.2	48.76
A/HK/400599/2008 (H1N1)	400599	Seasonal influenza	12 (43)	12 (43)	38.07	40.00

*GMT, geometric mean titer.
†Seroconverters were persons with antibody titer ≥40.

Conclusions

In this study, we focused on defining the effects of seroconversion to pandemic (H1N1) 2009 on serologic cross-reactivity to other swine subtype H1 viruses. The next step should be to ascertain herd immunity to these swine influenza viruses in different population groups. We chose not to do this at this stage because the pandemic virus is still circulating among human populations, and seroprevalence is likely to continue to increase in different age groups over the next few years. Therefore, studying the effect of seroconversion to pandemic (H1N1) 2009 on cross-reaction to other swine influenza viruses would provide more meaningful information at this stage.

The results of our study suggest that the spread of pandemic (H1N1) 2009 in the population is broadening the serologic cross-reactivity and immunity in humans to other swine influenza viruses. However, gaps in immunity to selected swine influenza subtype H1 viruses remain (e.g., Sw112), at least as ascertained by neutralization antibody titers. We recognize, however, that neutralization tests do not capture all aspects of herd immunity in a population. Thus, our findings only serve to focus attention on the need for further study of population immunity to viruses such as Sw112. In general, these findings highlight the need for enhanced global surveillance of swine influenza viruses for the systematic assessment of human herd immunity to novel swine strains and to facilitate the development of routine (evidence-based) procedures for the ranking of known strains in terms of their pandemic risk.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the US Department of Health and Human Services.

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Plasmodium knowlesi Infection in Humans, Cambodia, 2007–2010

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Two cases of *Plasmodium knowlesi* infection were identified in humans in Cambodia by 3 molecular detection assays and sequencing. This finding confirms the widespread distribution of *P. knowlesi* malaria in humans in Southeast Asia. Further wide-scale studies are required to assess the public health relevance of this zoonotic malaria parasite.

In Cambodia, malaria ranks among the leading causes of illness and death. Mostly affecting the ≈3 million persons (23% of Cambodia's population) who live near forested areas, malaria remains an occupational disease in specific high-risk groups, such as forestry workers and migrant populations who have come into forested areas. However, for the past decade, the number of reported malaria cases has generally decreased but in a sawtooth pattern of periodic increases (*1*).

Four of the 5 *Plasmodium* species known to cause malaria in humans have already been described in Cambodia (*2*,*3*). *P. falciparum* remains the most frequent cause of malaria (83,777 cases in 2009, prevalence of 70%) (*1*). However, distributions of *Plasmodium* species are changing, with a particularly substantial increase of *P. vivax* malaria cases, from 4,105 (8%) cases in 2000 to 6,250 (25%) in 2009. In several areas of low transmission, the proportion of *P. vivax* infections has increased up to 50% (*2*). This trend is probably related to various effective control strategies implemented in Cambodia against *P. falciparum* malaria.

No studies in humans (*3*) and monkeys in Cambodia have identified the simian malaria parasite, *P. knowlesi*, which is causing human disease in some other countries in Southeast Asia (*4*). *P. knowlesi* parasites were not detected in blood samples collected during 2004–2007 from 138 monkeys (102 *Macaca fascicularis* monkeys; 13 *M. leonina* monkeys; 20 *Hyobates pileatus* monkeys; 2 *Presbytis cristata* monkeys; and 1 *Pongo pygmaeus* monkey) by using PCR (*cytb* and *cox1* genes) (L. Duval, unpub. data). Because these animals were confiscated by Wildlife Alliance rescue patrols from illegal traders, the locations where they were trapped in Cambodia are unknown.

We undertook a cross-sectional prospective study in 3 sites in Cambodia (Figure, panel A) (*5*). The main objective of this study was to develop evidence to guide the management of malaria parasite–negative persons with acute febrile illness and to determine whether such persons were infected with *P. knowlesi*.

The Study

We enrolled patients ≥7 years of age with acute (<8 days) febrile illness in selected outpatient clinics, where they had been tested for malaria by using rapid diagnostic test (CareStart, Access Bio Inc, Somerset, NJ, USA). A subset of nonfebrile patients were used as controls; patients in a critical clinical condition were excluded. After we obtained informed consent, each patient's history was taken, a physical examination was conducted, and blood and throat swab samples were collected. The defined test battery, including the following pathogens, was performed by using a PCR/sequencing approach: *Plasmodium* spp., *Leptospira* spp., *Rickettsia* spp. (including *Orientia tsutsugamushi*), dengue viruses, Japanese encephalitis virus, and influenza viruses. HIV infection and tuberculosis were not evaluated. DNA and RNA from the erythrocyte pellet or throat swab were extracted by using the QIAamp kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Malaria parasites were detected by using a *Plasmodium* spp.–specific nested PCR based on the *cytb* gene followed by sequencing (*6*).

During December 2007–December 2010, a total of 1,475 patients were enrolled (621 in Soun Kouma; 650 in Ou Chra, Pailin Province; and 204 in Snoul, Kratié Province), comprising 1,193 febrile and 282 nonfebrile persons. For 564 (38.2%) patients, no pathogens were detected. A total of 754 patients (51.1%; 676 cases, 78 controls) were infected with malaria parasites, and the

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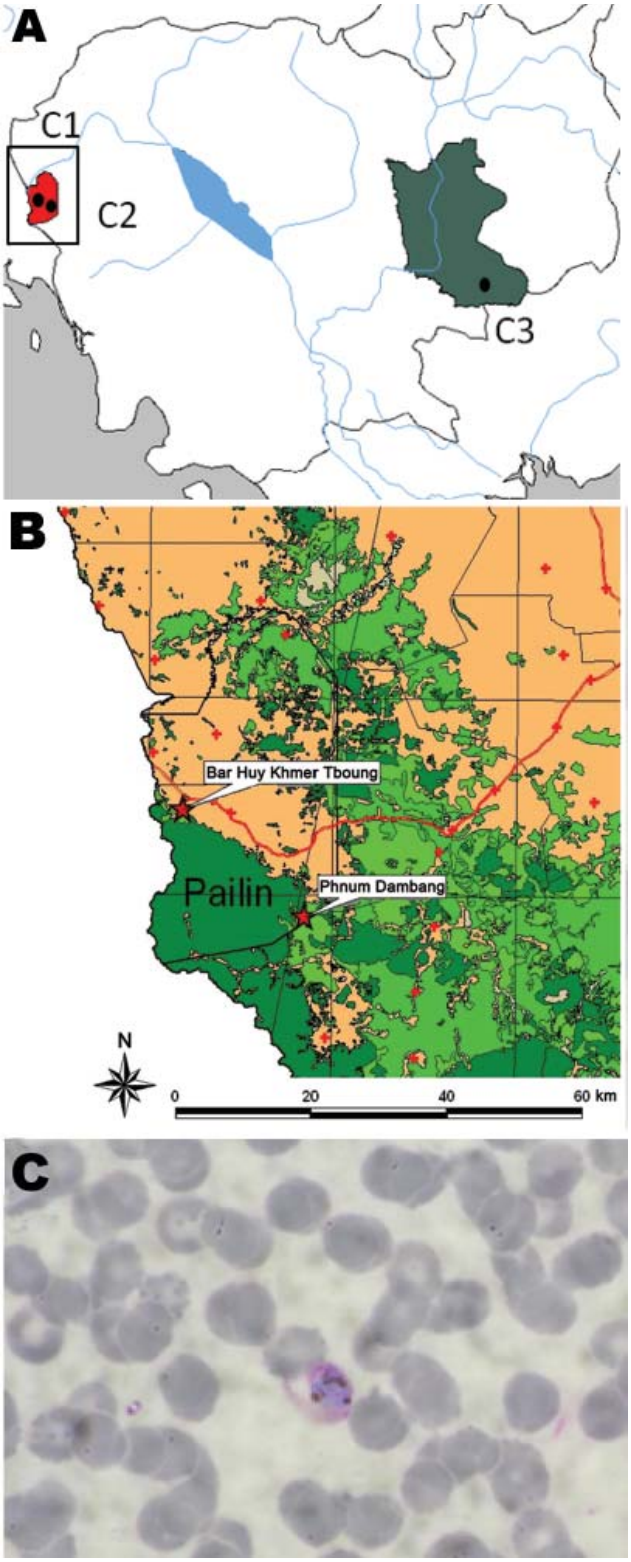

Figure. A) The 3 sites involved in the epidemiologic study of *Plasmodium knowlesi* malaria, Cambodia, 2007–2010. B) Villages in Pailin Province in which the 2 *P. knowlesi* malaria case-patients lived. C) Thin blood film slide of 1 PCR-confirmed *P. knowlesi* single infection (case C2611). Original magnification ×1,000.

distribution of *Plasmodium* spp. was as follows: *P. vivax*, 51.6%; *P. falciparum*, 40.6%; *P. vivax/P. falciparum*, 7.4%; *P. knowlesi*, 0.3%; and *P. ovale*, 0.1%.

We detected *P. knowlesi* infections in 2 patients from Ou Chra health center in Pailin Province (Figure, panel B). The first patient was a 41-year-old man from Borhuytbong village. He came in April 2010 to the health center with fever (38.5°C), chills, and headache. Rapid diagnostic test and microscopy were negative for malaria; PCR/sequencing was positive for *P. knowlesi*. No other pathogen was found. The patient was originally from Thailand and reported that he had not returned to Thailand since getting married in Cambodia in 1989. He reported self-treatment with mefloquine (Lariam [Roche, Basel, Switzerland]) and was cured in 3 days. He further added that he used to hunt and spent most of his time in forests around Pailin, where long-tailed macaques, the natural hosts of *P. knowlesi* (*7*), are usually found.

The second patient was a 40-year-old Khmer man from Phnomdambang village. He came in September 2010 to the health center because of chills and headache. No fever (36°C) was detected by the medical staff, but the patient reported a history of fever and had self-treated with acetaminophen. Results of rapid diagnostic test were negative, but microscopy results were positive (33 parasites/μL) (Figure, panel C). PCR amplification followed by sequencing confirmed *P. knowlesi* infection. No other pathogen was found. The patient reported having lived in this same village since 1995, and he frequently went to the forests around Pailin for hunting and collecting valued wood.

Both patients' *P. knowlesi* infections were confirmed by additional PCR amplification and sequencing of the *ldh*, *tufA*, and *cox* genes (*8*) at the Genomic Platform, Institut Pasteur, Paris, France. Blood spots on filter paper were also sent blinded to the Malaria Research Centre, Faculty of Medicine and Health Sciences, University Malaysia, Sarawak, Malaysia, where they were identified as *P. knowlesi* single infections by real-time PCR (*9*) and by nested PCR with the primers Pmk8 and Pmkr9 (*10*) and PkF1140 and PkR1550 (*11*). The nucleotide sequence determined in this study has been deposited in the GenBank database and assigned accession nos. JF419317–JF419323.

Conclusions

Our findings confirm that *P. knowlesi* infections occur in humans in Cambodia, thereby increasing the number of countries in Southeast Asia with cases in humans (*10*). However, further wide-scale studies are required to assess the prevalence and distribution of *P. knowlesi* malaria cases in humans and monkeys. Such studies would enable an assessment of the public health relevance of this zoonotic malaria parasite and characterization of *P. knowlesi* malaria

epidemiology in this region. They would be particularly useful in determining whether this simian species has been imported from neighboring countries by humans or whether *P. knowlesi* parasites are already circulating and are transmitted from monkey reservoir hosts to humans. Moreover, to address this issue, development of new tools, such as specific serologic markers, is urgently needed, in addition to molecular biology methods.

Acknowledgments

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Dr Khim is an engineer and head of the molecular biology platform in the Malaria Molecular Epidemiology Unit at Pasteur Institute of Cambodia. Her research interests include developing molecular tools for improving the surveillance of resistance to antimalarial drugs in Cambodia.

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Equine Piroplasmosis Associated with *Amblyomma cajennense* Ticks, Texas, USA

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We report an outbreak of equine piroplasmosis in southern Texas, USA, in 2009. Infection prevalence reached 100% in some areas (292 infected horses). *Amblyomma cajennense* was the predominant tick and experimentally transmitted *Theileria equi* to an uninfected horse. We suggest that transmission by this tick species played a role in this outbreak.

Theileria equi (*incertae sedis*; *Piroplasma equi* Laveran, 1901) is one of the etiologic agents of equine piroplasmosis. This parasite infects equids worldwide, but a few countries (Australia, Great Britain, Japan, United States, and Canada) are classified as free of this disease. These and several other countries restrict entry or internal movement of horses on the basis of their serologic response to *T. equi* antigen.

Particular tick species are obligate intermediate hosts and vectors for *T. equi* (*1*), which undergoes a complex developmental cycle in the vector similar to that of other apicomplexan hemoparasites (*2*,*3*). Asymptomatic persistent parasitemia detectable by serologic analysis or PCR develops in equids that survive acute infection. International movement of asymptomatic carriers poses a risk for introduction of equine piroplasmosis into regions free of this disease, but endemic transmission occurs only in regions that have competent vectors.

The World Organisation for Animal Health has listed the United States as free of equine piroplasmosis since 1978, although recent cases have occurred. Some of these cases may have resulted because the complement fixation test, formerly used for import screening, was not sufficiently sensitive to make a correct diagnosis. When transmission has occurred, it has been iatrogenic rather than vector-borne. Only 2 experimentally competent vectors of *T. equi* are known in the United States: *Dermacentor variabilis* (American dog tick) and *Rhipicephalus* (*Boophilus*) *microplus* (southern cattle tick) (*4*). However, of the 90 tick species in the United States, few have been tested for equine piroplasmosis vector competence (*1*,*4*).

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The Study

On October 2, 2009, a mare in Kleberg County, Texas, USA, showed clinical signs of equine piroplasmosis. Serologic testing at the Animal Plant Health Inspection Service, National Veterinary Service Laboratories (NVSL), US Department of Agriculture (USDA) (Ames, IA, USA) with a commercially available competititve ELISA (VMRD Inc., Pullman, WA, USA) detected *T. equi* antibodies. The remaining 359 horses on the index ranch were tested in the same way, and 292 (81.1%) of 360 were seropositive for *T. equi* on initial screening (Table 1).

Ticks collected from horses on the index ranch were shipped alive to NVSL. Identifications were made by using morphologic characteristics, geographic distribution, biologic characteristics, and host associations (*5*–*9*). NVSL received ticks from 228 horses; >1 species was present on 41 animals. The dominant tick, *Amblyomma cajennense*, was collected from 180 (78.9%) horses (Table 2).

All ticks were identified and sent to the Agricultural Research Service, Animal Disease Research Unit, USDA (Pullman, WA, USA) for transmission studies. Live males and partially fed females were pooled by species and held at 25°C and a relative humidity of 98% until they were allowed to reattach and feed on uninfected horses.

A total of 104 *A. cajennense* ticks (45 male and 79 female) were placed on a horse on October 30, 31, and November 2, 2009. These ticks had been removed from 73 horses on the index ranch, of which 68 (93.2%) were seropositive for *T. equi*. Females were allowed to reattach and feed until repletion; males were removed when all females were replete. All ticks were removed by November 18, 2009. Twenty-four fully engorged females and 3 live males were recovered. The horse had a fever (>39°C) 14 days after the ticks were first applied. Parasitized erythrocytes on a stained blood smear peaked at 0.3% on day 17. No other clinical signs of infection were evident. Serologic analysis and PCR (*10*) confirmed *T. equi* infection.

Twenty-nine *D. variabilis* ticks (12 male and 17 female) were placed on a second uninfected horse on October 30 and 31 and November 2 and 12, 2009. These ticks had been removed from 17 horses, of which 11 were seropositive and 1 was seronegative for *T. equi*; 5

Table 1. Horses tested by competitive ELISA for *Theileria equi* on index ranch of equine piroplasmosis outbreak in southern Texas, USA, 2009

Ranch division	No. positive/no. tested (%)
A*	213/281 (75.8)
B	36/36 (100)
C	10/10 (100)
D	33/33 (100)
Total	292/360 (81.1)

*Division A contained all younger stock. Infection rates among younger animals were lower. The other 3 divisions contained mostly horses used for working cattle.

had an unknown infection status. All ticks were removed by November 24, 2009. Six fully engorged females and 7 live males were recovered. This horse had a slight fever (39°C) 15 days after tick attachment but otherwise showed no clinical signs. No organisms were found in blood smears, but this horse was positive for *T. equi* by PCR 42 days after the first ticks were attached and by competitive ELISA 87 days after tick attachment.

Conclusions

Ranch staff reported that they used no practices that would result in movement of blood-contaminated materials between horses (e.g., no reuse of needles), which suggests that iatrogenic transmission was not responsible for this outbreak. Consequently, the high prevalence of *T. equi* infection implies a focus of vector-borne transmission.

A. cajennense ticks were the most abundant species on horses during the period (October–November) of this investigation (Table 2). Our results demonstrate that *A. cajennense* ticks naturally acquired infection while feeding on infected horses and transmitted *T. equi* intrastadially when they reattach and feed on uninfected hosts. *A. cajennense* ticks have not been shown experimentally to be a competent vector for *T. equi* (*1*). This species is a 3-host tick, and all life stages are known to feed aggressively on a wide variety of hosts, including horses. The natural distribution of *A. cajennense* ticks includes southeast Texas; they are not known to be present in other parts of the United States where cases of equine piroplasmosis have occurred (*11*). Although this study demonstrates that *A. cajennense* ticks are an experimental intrastadial vector, additional studies are needed to fully characterize the vector capacity of this species, particularly with regard to interstadial transmission.

Immature stages of *D. variabilis* ticks occur almost exclusively on rodents; only adults were found on horses at the ranch. Although these ticks were able to transmit *T. equi* intrastadially to an uninfected horse, the small proportion of infested horses on the ranch (16.2%; Table 2) and low transmission efficiency of this species (G.A. Scoles, unpub. data) make it unlikely that *D. variabilis* ticks were responsible for the high infection prevalence.

A. maculatum, the Gulf Coast tick, was the second most abundant species on horses at the index ranch during the study (19.7%; Table 2). However, this species survived poorly during handling and transport, probably because it is less tolerant of desiccation than are *A. cajennense* ticks (*12*), and we did not have enough viable ticks to attempt transmission feeding. Whether this species can act as a vector for *T. equi* is unknown. *D.* (*Anocentor*) *nitens* ticks were collected from 7 (3%) horses sampled. This species is a proven vector of *Babesia caballi* but has not been shown to be a vector of *T. equi*. *R. microplus* ticks are limited to a quarantine zone along the Texas–Mexico border. The index ranch is north of this zone, and no *R. microplus* ticks were found on horses at this ranch.

Although *T. equi* can be transmitted iatrogenically, e.g., by common needle use (*13*), this route of transmission is improbable with good management practices. Vector-borne transmission is more likely than iatrogenic transmission to establish and maintain a large focus of infection, such as in this outbreak. Additional tick studies are needed to determine whether other indigenous tick species are involved in transmission at this site. However, if *A. cajennense* ticks are the primary vector, the outbreak will likely be confined to this region because southeastern Texas is the northern extent of the range of this tick in the United States (*11*). Given knowledge of tick species that are competent vectors, spread of this parasite can be controlled by testing requirements and limits on regional movement of equines on the basis of presence or absence of such competent vectors.

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Table 2. Tick species found on horses at index ranch of equine piroplasmosis outbreak in southern Texas, USA, 2009

Species	No. (%) horses*	No. ticks			Average no. ticks/horse
		Male	Female	Nymph	
Amblyomma cajennense	180 (78.9)	201	229	1	2.4
A. maculatum	45 (19.7)	43	33	0	1.7
Dermacentor (*Anocentor*) *nitens*	7 (3.0)	4	7	3	2
D. variabilis	37 (16.2)	20	34	0	1.6

*Of 228 horses sampled, 41 had >1 species of tick present.

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Timeliness of Surveillance during Outbreak of Shiga Toxin–producing *Escherichia coli* Infection, Germany, 2011

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In the context of a large outbreak of Shiga toxin–producing *Escherichia coli* O104:H4 in Germany, we quantified the timeliness of the German surveillance system for hemolytic uremic syndrome and Shiga toxin–producing *E. coli* notifiable diseases during 2003–2011. Although reporting occurred faster than required by law, potential for improvement exists at all levels of the information chain.

In May and June 2011, Germany experienced the largest outbreak of hemolytic uremic syndrome (HUS) and bloody diarrhea related to Shiga toxin–producing *Escherichia coli* (STEC) ever reported (*1*,*2*). As of June 20, a total of 2,518 STEC cases and 786 HUS cases meeting the case definitions for this outbreak were reported to the national agency for infectious disease epidemiology (Robert Koch Institute [RKI]) through the surveillance system in Germany for notifiable diseases (GSSND) (*3*). The first outbreak-associated case-patient fell ill on May 1, followed by a sharp increase in the number of HUS case-patients on May 9 (by onset of symptom). Case numbers by disease onset peaked around May 22. Epidemiologic and food trace-back investigations identified fenugreek sprouts, grown from seeds probably contaminated by STEC, at a farm in Lower Saxony to be the vehicle of the outbreak (*4*). From June 10 on, German authorities recommended that raw sprouts should not be eaten.

In the GSSND, heads of laboratories have to send notification of STEC cases, and clinicians are legally mandated to report HUS cases within 24 hours to the local health department by fax, telephone, or letter (*5*). Legally, reporting of these cases from the local health department through the state health department to RKI must be completed within 16 days. To minimize the reporting delay, beginning May 23, the local health departments and state health departments agreed to report cases every working day (*6*).

On May 18, the first outbreak-associated case (patient's onset of diarrhea was May 2) was reported to RKI. On the same day, a local hospital notified the local health department of Hamburg North about a cluster of HUS in 3 children. RKI was alerted to the outbreak cluster on May 19 by email.

Given the extent of the outbreak, questions arose about the timeliness of the GSSND regarding STEC and HUS cases. We assessed intervals between notifying and reporting STEC and HUS cases from January 1, 2003, through June 22, 2011, to identify potential needs and strategies for improvement.

The Study

We divided the analysis into 3 periods: period A (before the outbreak) was from January 1, 2003, through April 30, 2011; period B (early phase of the outbreak) was from May 1 (when the first outbreak-associated case-patient fell ill) through May 18 (date when the HUS cluster was detected); and period C (late phase of the outbreak) was from May 19 through June 22. Data on timeline events for all reported STEC and HUS cases were collected from the GSSND (*7*). Timeline events comprised the following dates: symptom onset (onset of diarrhea), diagnosis, notification (date when the notification arrived at the local health department), and reporting (date when the report arrived at RKI). Dates of symptom onset and diagnosis were excluded when they were after the date of notification. For each case, intervals between timeline events were calculated from the dates available. Intervals were then assigned to 1 of the 3 periods (A, B, or C) according to the first date of the interval. Median times and interquartile ranges (IQR) were calculated in days for each type of interval for each period. Statistical analyses were done by using Stata software version 11.0 (StataCorp LP, College Station, TX, USA).

For the 1,394 HUS cases with available information, the median times from symptom onset in patients to diagnosis and to notification were similar in periods A and B (8 days and 9–10 days, respectively) and shorter in period C (4 and 5 days, respectively) (Table 1). The median time from symptom onset to reporting decreased from 20 days in period A to 12 and 8 days in periods B and C, respectively. The median time from diagnosis to notification was longer in period B (4.5 days) than in periods A and C (1 and 0 day, respectively). Among the 14 HUS cases with available information for period B, 10 (71%) were notified after 24 hours. The interval from notification of the local health

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Table 1. Median reporting intervals, in days, for 1,394 hemolytic uremic syndrome cases in 3 periods, Germany*

	Period A		Period B		Period C		
Interval†	No./N‡ (%)	Med (IQR)	No./N‡ (%)	Med (IQR)	No./N‡ (%)	Med (IQR)	Total no./N‡ (%)
From symptom onset to							1,267/1,394§ (91)
Diagnosis	283/497 (57)	8 (4–12)	136/237 (57)	8 (6–9)	331/533 (62)	4 (2–6)	750/1,267 (59)
Notification to LHD	312/497 (63)	10 (6–15)	173/237 (73)	9 (7–10)	396/533 (74)	5 (3–7)	881/1,267 (70)
Report to RKI	497/497 (100)	20 (14–26)	237/237 (100)	12 (9–15)	533/533 (100)	8 (6–11)	1,267/1,267 (100)
From diagnosis to							798/1,394§ (57)
Notification to LHD	264/294 (90)	1 (0–3)	14/15 (93)	4.5 (1–7)	473/489 (97)	0 (0–1)	751/798 (94)
Report to RKI	294/294 (100)	10 (7–16)	15/15 (100)	8 (8–9)	489/489 (100)	3 (2–6)	798/798 (100)
From notification to LDH to							943/1,394§ (68)
Report to RKI	319/319 (100)	7 (5–13)	6/6 (100)	8 (6–8)	618/618 (100)	3 (1–5)	943/943 (100)

*Period A, 2003 Jan 1–2011 Apr 30; Period B, 2011 May 1–2011 May 18; Period C, 2011 May 19–2011 Jun 22; med, median; IQR, interquartile range; LHD, local health department; RKI, Robert Koch Institute.
†Classification of the interval in 1 of the 3 periods according to the first date of this interval.
‡No./N, no. patients having available data for both dates of the interval/no. patients having available data for the first date of the interval.
§No. patients having available data for this date/total no. hemolytic uremic syndrome cases.

department to report to RKI was longer in periods A and B than in period C (7 and 8 days vs. 3 days, respectively). For the 13,400 STEC cases with available information, we noticed in period B a longer delay from symptom onset in patients to reporting (15 days vs. 12 days for HUS) but a shorter delay from diagnosis to notification (2 days vs. 4.5 days for HUS) (Table 2). The Figure shows the increasing numbers of patients with disease onset on May 9, diagnosis and notification on May 18, and reporting on May 24.

Conclusions

A median of 11 days passed between onset of symptoms and notification of STEC cases in period A. A study by Hedberg et al. in 6 US states reported a delay of 7 days for the same interval for *E. coli* O157 infections (*8*). Considering that period B is biased for the interval "onset of symptom to reporting," because the second date of the interval is likely to be in period C when the reporting flow was accelerated, we could only consider period A for this interval. We found that a median of 20 days occurred between symptom onset and reporting for STEC and HUS cases. This result is comparable to the 18 days reported for foodborne infections in the Netherlands (*9*). However, the duration between symptom onset and reporting can be reduced to 8 days, as was seen in period C. We also found that most of the HUS cases in period B were notified later than mandated by law. Although the number of cases was small, this is a remarkable result. It might be explained by the limited experience of nephrologists in notifying adult HUS cases. However, this also shows the need to motivate and to assist clinicians to notify within 24 hours (e.g., with an automatic electronic notification tool that could alert clinicians of their obligation to notify the disease when entering the diagnosis of HUS). By looking only at the timeline events directly under control of public health authorities, we found that the interval from

Table 2. Median reporting intervals, in days, for 13,400 Shiga toxin–producing *Escherichia coli* cases in 3 periods, Germany*

	Period A		Period B		Period C		
Interval†	No./N‡ (%)	Med (IQR)	No./N‡ (%)	Med (IQR)	No./N‡ (%)	Med (IQR)	Total no./N‡ (%)
From symptom onset to							9,365/13,400§ (70)
Diagnosis	4,734/6,700 (71)	8 (5–16)	368/494 (74)	9 (6–13)	1,606/2,171 (74)	4 (3–6)	6,708/9,365 (72)
Notification to LHD	4,652/6,700 (69)	11 (7–18)	423/494 (86)	10 (7–15)	1,848/2,171 (85)	5 (3–7)	6,923/9,365 (74)
Report to RKI	6,700/6,700 (100)	20 (14–30)	494/494 (100)	15 (11–20)	2,171/2,171 (100)	9 (6–12)	9,365/9,365 (100)
From diagnosis to							9,261/13,400§ (69)
Notification to LHD	6,088/6,802 (90)	1 (0–3)	69/70 (99)	2 (0–6)	2,353/2,389 (98)	0 (0–1)	8,510/9,261 (92)
Report to RKI	6,802/6,802 (100)	9 (6–14)	70/70 (100)	9.5 (7–13)	2,389/2,389 (100)	4 (2–6)	9,261/9,261 (100)
From notification to LDH to							9,529/13,400§ (71)
Report to RKI	6,712/6,712 (100)	7 (4–11)	50/50 (100)	8 (6–11)	2,767/2,767 (100)	3 (1–5)	9,529/9,529 (100)

*Period A, 2003 Jan 1–2011 Apr 30; Period B, 2011 May 1–2011 May 18; Period C, 2011 May 19–2011 Jun 22; med, median; IQR, interquartile range; LHD, local health department; RKI, Robert Koch Institute.
†Classification of the interval in 1 of the 3 periods according to the first date of this interval.
‡No./N, no. patients having available data for both dates of the interval/no. patients having available data for the first date of the interval.
§No. patients having available data for this date/total number of Shiga toxin–producing *Escherichia coli* cases.

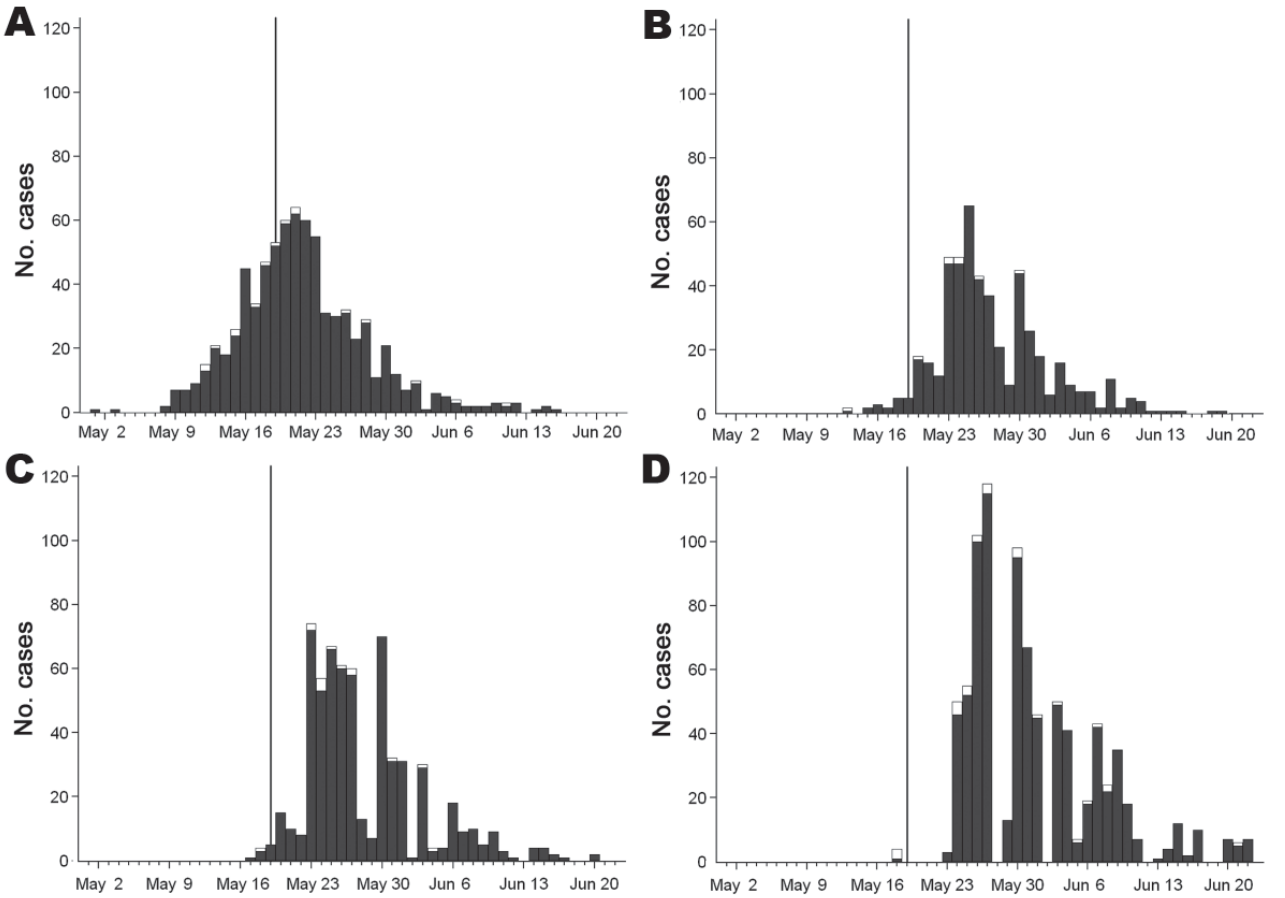

Figure. Hemolytic uremic syndrome (HUS) cases by date of symptom onset (A), date of diagnosis (B), date of notification (C) (i.e., the date that the local health department was notified of the case), and date of reporting (D) (i.e., the date that the Robert Koch Institute received the report of the case from the local health department) during outbreak of Shiga toxin–producing *Escherichia coli* infection and HUS, May–June 2011, Germany. Vertical lines indicate May 19, when the Robert Koch Institute received reports about a cluster of HUS cases in children. Dark gray bars represent outbreak-related cases; white bars represent cases not related to the outbreak. Only cases with available information are represented.

notification of the local health department to reporting to RKI could be shortened from 1 week to 3 days if the local health department and the state health department routinely transmitted data on a daily basis.

This outbreak is a good example of circumstances in which single cases occur initially in multiple local health administrations in different federal states. In such situations, early outbreak detection and investigation become crucial to ensure early and continuous reporting to authorities at the national level. Given the current delays in diagnosis, notification, and reporting, this outbreak would have been detected at the national level considerably later than May 19 if the Hamburg health department had not promptly contacted RKI. This illustrates that state health departments and RKI need to receive local notifications earlier to successfully apply detection algorithms that would indicate potential multicounty or multistate outbreaks (*10*).

A revision of the notification and reporting system should be considered in Germany, with the goal of timely detection of increases in infectious diseases while being sustainable and specific. This result could be achieved if physicians and heads of laboratories could feed their data into a centralized database shared by local health departments, state health departments, and RKI with different access rights.

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Global Distribution of *Shigella sonnei* Clones

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To investigate global epidemiology of *Shigella sonnei*, we performed multilocus variable number tandem repeat analysis of 1,672 isolates obtained since 1943 from 50 countries on 5 continents and the Pacific region. Three major clonal groups were identified; 2 were globally spread. Type 18 and its derivatives have circulated worldwide in recent decades.

Shigella sonnei is the most commonly isolated species among the 4 *Shigella* species in industrialized countries (*1*,*2*). Transmission of *S. sonnei* across geographic boundaries is frequently linked to international travel and cross-border food trade (*3*,*4*). *S. sonnei* is a monomorphic organism and therefore requires a highly discriminative sequence-based method for investigating its clonal structure and the geographic distribution of clones.

A total of 26 variable number tandem repeats (VNTRs) have been used to type *S. sonnei* isolates (*5*). Because VNTRs have a wide range of variability, they are useful markers for investigating clonal relationships among strains that have evolved over different times (*6*). In this study, we analyzed 1,672 *S. sonnei* isolates obtained since 1943 from 50 countries on 5 continents (Africa, Asia, Europe, North America, and South America) and the Pacific region by multilocus VNTR analysis (MLVA) to investigate the global epidemiology of *S. sonnei*.

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The Study

Isolates were obtained from 50 countries on 5 continents and the Pacific region (online Appendix Table, www.cdc.gov/EID/content/17/10/10-1486-appT.htm). Of these isolates, 31 were obtained during 1943–1983 (from Cameroon, Denmark, France, Senegal, and Sweden) and 1,641 were obtained during 1994–2008 from 48 countries. Isolates were lyophilized, kept in stab culture medium, or stored in 15%–20% glycerol at –75°C for long-term storage. The isolates were not repeatedly subcultured before this study. MLVA26, an MLVA assay based on analysis of 26 VNTRs, classified the 1,672 isolates into 620 MLVA26 types. With only 2 exceptions, no common MLVA26 type was shared among isolates from different countries. One isolate from Malaysia (1999) and 2 isolates from Vietnam (2006) shared a common MLVA26 type, and an isolate from Chad and an isolate from France (both isolated in 2007) shared a common MVLA26 type.

The high resolving power of MLVA is primarily caused by highly diverse VNTRs (*6*). Clustering analysis of the MLVA26 types using a minimum spanning tree (MST) algorithm grouped the 1,672 isolates into 3 large clusters (A, B, and C), 1 small cluster (D), and 1 singleton (E). Each cluster was defined to include MLVA26 types differing at ≤7 loci among the 26 loci. The 3 large clusters displayed distinct allelic diversity features. Eight loci (SS1, SS3, SS6, SS9, SS10, SS11, SS12, and SS23) had Simpson diversity values >0.5 for ≥1 of the 3 large clusters (Table). Differences in diversity values >0.3 among the 3 clusters were observed for 9 of the 26 VNTRs. The largest difference was in 2 hypervariable VNTRs (SS1 and SS6). These 2 VNTRs displayed a high degree of allelic diversity in cluster A, but were invariant in cluster B. SS1 was invariant but SS6 displayed a high degree of diversity in cluster C.

Of 1,672 isolates, 66% (1,100) were obtained from patients who acquired infections in Taiwan. Most isolates belonged to an insertion element IS1 interspacer 1 clone (*6*,*7*) that had slightly lower diversity values for some VNTRs than diversity values for total isolates obtained from a panel of 620 isolates representing the 620 MLVA26 types. However, a large number of clonal isolates from Taiwan did not affect relative magnitudes of diversity of the 26 VNTRs.

Although highly variable VNTRs are useful markers in distinguishing closely related strains, they are less useful for investigating clonal relationships among strains that have evolved over time (*6*). MLVA18 profiles, which excluded the data of 8 highly variable VNTRs (SS1, SS3, SS6, SS9, SS10, SS11, SS12, and SS23) from the 26-locus panel, were used to investigate the clonal structure of the isolates. On the basis of 18-locus profiles, 105 MLVA18 types were identified.

Table. Allelic diversity and range of repeats of VNTRs for 3 major *Shigella sonnei* clonal groups*

VNTR	Repeat unit, bp	No. alleles	Clonal group A, n = 1,382		Clonal group B, n = 75		Clonal group C, n = 212	
			Allelic diversity†	Range of repeats	Allelic diversity	Range of repeats	Allelic diversity	Range of repeats
SS1	7	16	0.71	1–16	0	1	0	1
SS2	9	3	0.02	2–3	0	2	0.04	1–3
SS3	7	30	0.88	2–29	0.90	2–34	0.82	2–28
SS4	7	3	0.01	2–6	0	2	0.10	2–3
SS5	7	3	0.08	2–4	0.03	2–3	0	3
SS6	7	29	0.85	2–31	0	2	0.86	4–30
SS7	7	3	0.49	2–3	0	2	0.02	2–3
SS8	60	3	0	1	0.10	1–2, 330‡	0.45	1–2
SS9	6	16	0.65	2–18	0.83	5–18	0.77	2–18
SS10	6	9	0.57	2–10	0.46	3–8	0.53	0, 3–8
SS11	6	8	0.63	2–9	0.64	2–8	0.65	3–9
SS12	9	5	0.01	2–3	0.58	2–6	0.18	2–4
SS13	6	5	0.19	2–6	0.03	0, 2	0.07	2–4
SS14	9	4	0.01	2–3	0	2	0.37	2–6
SS15	6	3	0.04	2–3	0.21	3–4	0.02	2–3
SS16	17	3	0.07	1–2	0	2	0.06	1–3
SS17	6	2	0	0, 2	0.15	2–3	0.04	2–3
SS18	5	3	0.01	2–3	0	2	0.30	2–4
SS19	5	2	0.03	2–3	0.23	2–3	0.03	2–3
SS20	40	2	0.04	1–2	0	1	0	1
SS21	18	3	0.01	0, 1–2	0.12	1–2	0.24	1–4
SS22	11	2	0.01	1–2	0.03	1–2	0	1
SS23	16	8	0.03	0, 2–6	0.55	0, 2–5	0.69	0, 2–10
SS24	168	2	0.06	0, 1–2	0.03	0, 1	0.05	1–2
SS25	135	2	0.01	1–2	0.03	1–2	0.41	1–2
SS26	101	5	0.01	2–5	0.03	1–5	0	4

*VNTR, variable number tandem repeat.
†Simpson's diversity index = $1 - \Sigma(n/N)^2$.
‡Allele 330 for SS8, which has an imperfect repeat, is designated by the size of amplicon instead of the number of repeats.

A simplified MST was created by analysis of a subset of 200 isolates selected by obtaining 1 MLVA18 type among those identified in each of 50 countries. As shown in the MST (Figure), cluster A was further divided into subclusters A1 and A2 and singleton A3, and cluster C was divided into subclusters C1 and C2.

Cluster A consisted of 46 MLVA18 types, which represented 1,382 isolates obtained since 1943 in 40 countries on 5 continents and the Pacific region (online Appendix Table). Several MLVA18 types within cluster A were widespread. SS18.2 was detected in 23 countries on 5 continents. SS18.2 had 14 single-locus variants (SLVs) at which genotypes differed only at 1 of the 18 loci; 3 (SS18.80, SS18.1, and SS18.60) of the SLVs were detected in 13, 10, and 7 countries, respectively. SS18.2 and its SLVs represented 1,290 isolates obtained in during 1995–2008 from 38 countries on 5 continents. Four MLVA18 types were detected in samples obtained in 1943–1983. These isolates shared identical MLVA18 profiles or differed at 1–2 loci from recently obtained isolates. Subcluster A2 consisted of 4 MLVA18 types found in isolates from Argentina only. Singleton A3, which was found in isolates obtained in Vietnam in 2008, was distantly separate from subclusters A1 and A2.

Cluster B consisted of 12 MLVA18 types representing 75 isolates, which were obtained in 8 countries in Africa, Asia, and Europe. SS18.6 had the highest number of SLVs in cluster B and was detected in 5 countries in Asia and Africa. Five types were detected in isolates obtained in 1943–1974; they shared identical MLVA18 profiles or differed at 1–2 loci from recently recovered isolates.

Cluster C was relatively diverse; it consisted of 44 MLVA18 types, which represented 212 isolates obtained in 21 countries on 5 continents and the Pacific region. SS18.8 had the highest number of SLVs and was found in isolates obtained during 1974–2007 in 8 countries on 5 continents. SS18.4, the largest SLV of SS18.8, was found in isolates from 5 countries on 5 continents. Subcluster C2 consisted of isolates from Argentina obtained in 2002 and Sweden and Denmark in 1943. These isolates emerged in 1943–1974 and were genetically similar to recently obtained isolates. Clusters (clonal groups) A and C were globally spread, and clonal group B was found in countries in Africa, Asia and Europe only.

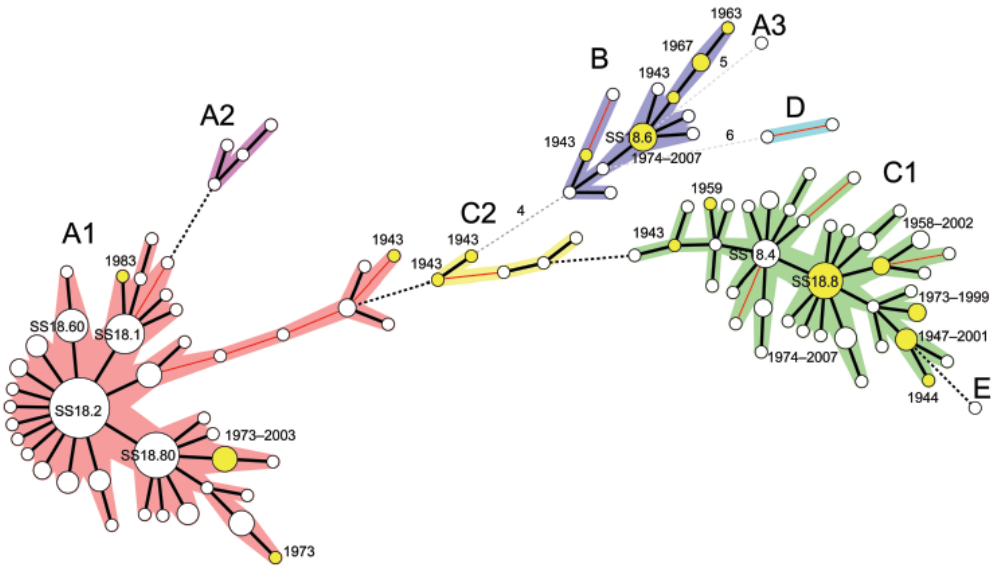

Figure. Clonal structure of 200 *Shigella sonnei* isolates. These isolates, representative of the 1,672 isolates analyzed in this study, were selected by obtaining 1 isolate for 1 multilocus variable number tandem repeat analysis (MLVA) 18 type from those identified in each of 50 countries on 5 continents (Africa, Asia, Europe, North America, and South America) and the Pacific region. The tree was constructed by using MLVA18 profiles and a minimum spanning tree algorithm. Circle size is proportional to the number of countries detected with the MLVA18 type. Genotypes in yellow indicate isolates obtained in the early period (1943–1983). A cluster or subcluster containing ≥2 genotypes differing at ≤2 loci is indicated by the 5 other colors. Distances of 1 locus between 2 closest genotypes are indicated by thick black lines, distances of 2 loci are indicated by thin red lines, distances of 3 loci are indicated by black dashed lines, and distances ≥4 loci are indicated by grey dashed lines. Numbers of different loci are indicated.

Cluster D contained 2 isolates obtained in French Guiana and Senegal in 2003. The isolate for singleton E was obtained in Malaysia in 1999.

Conclusions

Genetic analysis using MLVA presented a simple clonal structure for 1,672 *S. sonnei* isolates obtained since 1943 from 50 countries on 5 continents and the Pacific region. Three large clonal groups were identified; they displayed distinct allelic diversity features, particularly for 2 hypervariable VNTRs (SS1 and SS6). Clonal groups A and C were globally spread. One MLVA18 type (SS18.2) and several of its SLVs were widely distributed over 5 continents in the past 10 years.

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Drug-Resistant Tuberculosis, KwaZulu-Natal, South Africa, 2001–2007

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In Africa, incidence and prevalence of drug-resistant tuberculosis have been assumed to be low. However, investigation after a 2005 outbreak of extensively drug-resistant tuberculosis in KwaZulu-Natal Province, South Africa, found that the incidence rate for multidrug-resistant tuberculosis in KwaZulu-Natal was among the highest globally and would be higher if case-finding efforts were intensified.

In Africa, resistance to anti-tuberculosis (TB) drugs has been assumed to be low (*1*). In 2002, the national drug resistance survey showed rates of multidrug-resistant (MDR) TB in South Africa to be 3.0% among all TB cases, 1.6% among new cases, and 6.6% among previously treated cases (*2*). Surveys in other African countries have yielded MDR TB rates of <3% among all TB cases, low compared with ≥20% reported from former Soviet Union countries; however, MDR TB rates may not be as low as previously estimated (*3*–*7*). In response to a 2005 outbreak of extensively drug-resistant (XDR) TB in KwaZulu-Natal Province, South Africa (*8*), we conducted a retrospective study of the extent and distribution of drug-resistant TB in the province.

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The Study

KwaZulu-Natal Province, population ≈10 million, contains 11 health districts with 68 hospitals in the public health sector. We analyzed existing laboratory records from the only 2 laboratories in the province that conducted culture and drug-sensitivity testing as part of routine clinical care. Since 2001, all samples from patients with MDR TB in the province were tested for susceptibility to second-line anti-TB drugs, except in 2004 and 2005, when 82% (1,143) and 55% (1,277) of samples, respectively, were not tested for fluoroquinolones. From 2006 on, all culture-positive cases in the province were tested for susceptibility to first-line and second-line anti-TB drugs. We reviewed laboratory results from 2001 through 2007 and determined the number of MDR TB and XDR TB cases for each district. To provide context for the 2005 XDR TB outbreak at the Church of Scotland Hospital (COSH) in KwaZulu-Natal Province (*8*), we also analyzed geographic distribution and time trends. Per definition, the number of MDR TB cases includes all XDR TB cases.

According to national guidelines, samples for culture should be collected from persons who are being initially examined for retreatment, those for whom treatment has failed, those whose sputum smear results are negative but who have clinical signs of TB and do not respond to antibacterial drug treatment (excluding TB treatment), and those suspected of having HIV and TB co-infection. Despite the guidelines, the intensity with which cultures were requested varied among districts. We compared culture-taking practices with prevalence of MDR TB per district during the same time frame. Culture-taking practices were derived from the number of patients for whom a culture was requested during 12 months after March 2006 and were analyzed per district and per 100,000 population. The proportion of identified MDR TB patients who received treatment with second-line anti-TB drugs was calculated by dividing the number of patients with laboratory-confirmed MDR TB by the number of patients admitted to King George V Hospital, the only TB hospital in the province that treated patients with MDR TB during the same period. (Detailed methods available from K.W. upon request.)

In 2007, a total of 2,799 cases (28 cases/100,000 population) of MDR TB were identified in KwaZulu-Natal. TB prevalence was 1,200 cases/100,000 population, and MDR accounted for 2.3% of reported cases in the province (http://arxiv.org/abs/1107.1800). XDR TB cases accounted for 9.6% of MDR TB cases (Table).

In 2007, MDR TB in the districts ranged from 10 (uThukela) to 57 (Umkhanyakude) cases per 100,000 population (Table). Incidence of MDR TB was highest for Umkhanyakude and Umzinyathi districts (location of COSH) (*8*). The proportion of MDR TB cases that were XDR TB cases also varied among districts (1.2%–

Table. Cases of MDR and XDR TB, KwaZulu-Natal Province, South Africa, 2007*

District	All TB cases	MDR TB cases	XDR TB cases	MDR/all TB cases, %	XDR/MDR cases, % (95% CI)	No. MDR cases/ 100,000 population
eThekwini	45,019	1,014	64	2.3	6.3 (4.9–8.0)	31.7
Ugu	10,618	226	9	2.1	4.0 (1.8–7.4)	31.0
uMgungundlovu	10,687	247	36	2.3	14.6 (10.4–19.6)	25.7
uThukela	6,129	69	8	1.1	11.6 (5.1–21.6)	10.1
Umzinyathi	5,522	226	120	4.1	53.1 (46.4–59.7)	47.8
Amajuba	3,578	61	2	1.7	3.3 (0.4–11.3)	12.6
Zululand	8,478	171	6	2.0	3.5 (1.3–7.5)	20.5
Umkhanyakude	6,991	337	4	4.8	1.2 (0.3–3.0)	56.8
Uthungulu	11,876	233	11	2.0	4.7 (2.4–8.3)	25.4
iLembe	5,007	118	6	2.4	5.1 (1.9–10.7)	20.3
Sisonke	5,313	61	4	1.1	6.6 (1.8–15.9)	12.9
Total	119,218	2,799	270	2.3	9.6 (8.6–10.8)	28.2

*MDR, multidrug-resistant; XDR, extensively drug-resistant; TB, tuberculosis; CI, confidence interval.

53.1%). The 2 districts with the highest level of MDR TB (Umkhanyakude and Umzinyathi) had the highest and the lowest XDR TB prevalence, respectively (Table).

From 2001 to 2007, the level of MDR TB increased >10-fold, from 216 cases to 2,799 cases, respectively (Figure 1, panel A), and XDR TB increased from 6 cases to 270 cases, respectively (Figure 1, panel B). In part, the increase reflects increased sampling, which tripled between 2002 and 2007, from ≈40,000 to >120,000 samples tested each year. The variation in MDR TB prevalence between districts could be attributed to differences in culture-taking practice (Spearman correlation coefficient 0.82; p = 0.001) (Figure 2). XDR TB prevalence, expressed as proportion of MDR TB, was not affected by culture-taking practices because all positive cultures were tested for first-line and second-line anti-TB drugs.

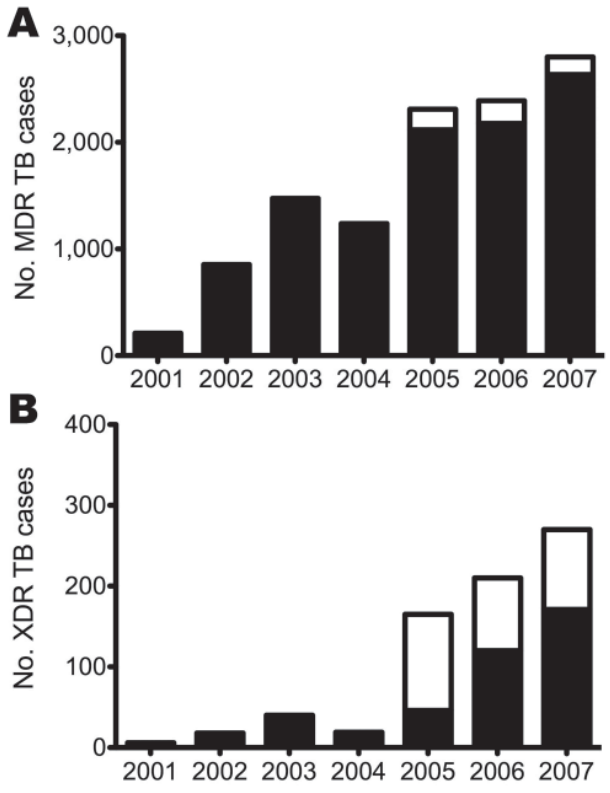

Figure 1. Prevalence of drug-resistant tuberculosis (TB) in the 11 districts of KwaZulu-Natal, South Africa, 2001–2007. A) Multidrug-resistant (MDR) TB; B) extensively drug-resistant (XDR) TB. White bar sections, Church of Scotland Hospital; black bar sections, the rest of KwaZulu-Natal Province.

XDR TB has been in KwaZulu-Natal Province since 2001 and was first identified in eThekwini. XDR TB increased rapidly in 2005 when the outbreak was identified, and 72% of all XDR TB cases in the province were at COSH (Figure 1, panel B). As a result of this outbreak, culture-taking practices in the rest of the province increased, and in 2007, the proportion of identified XDR TB cases in the province that were at COSH had decreased to 37%. Excluding Umzinyathi, an average of 6.5% of all MDR TB cases were also XDR TB.

Within 12 months after March 2006, only 32% (896) of 2,784 patients with laboratory-identified MDR TB had received treatment with second-line drugs. During 2005–2006, the average time between sputum collection and admission to King George V Hospital was 16 weeks. The delay reflects turnaround time for culture and sensitivity testing (4–6 weeks), delay in returning results to the referring health facility (not all sites have Internet access), tracing of patients, and hospital admission waiting time.

Conclusions

In 2007, South Africa ranked fourth among countries with the highest estimated number of MDR TB cases (*9*). Within South Africa, KwaZulu-Natal Province had the highest prevalence of drug-resistant TB and accounted for 38% (2,799) of 7,350 MDR TB cases and 50% (270) of 536 XDR TB cases in the country (*10*,*11*).

The reported MDR TB incidence rate per 100,000 population of KwaZulu-Natal is among the highest worldwide. Districts Umkhanyakude and Umzinyathi reported 57 and 48 cases/100,000 population, respectively,

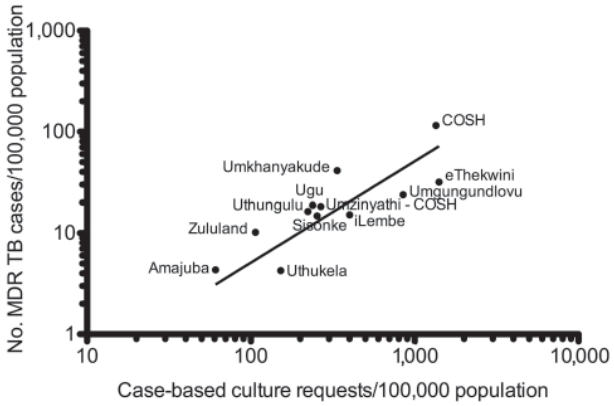

Figure 2. Culture-taking practice correlation with identified multidrug-resistant tuberculosis (MDR TB) prevalence in the 11 districts of KwaZulu-Natal Province and in the Church of Scotland Hospital (COSH), South Africa, 2001–2007. Because of the high level of culture-taking at COSH, COSH data were subtracted from the Umzinyathi district data. Black line indicates the level of MDR TB that would be identified if the whole province requested the same number of culture and sensitivity testing as COSH.

more than the highest previously reported estimate of 35 cases/100,000 population in Karakalpakstan, Uzbekistan (*12*). Because many patients never have a sample taken for culture and sensitivity testing, the identified level of MDR TB is an underestimate.

After the 2005 outbreak, COSH increased vigilance for drug resistance, and culture and sensitivity testing was conducted for all patients with suspected TB. If other clinics and hospitals in the province requested as many cultures as COSH, MDR TB in KwaZulu-Natal would amount to 68 cases/100,000 population, with an estimated 6,750 MDR TB cases and 526 XDR TB cases per year, 2–3× more than currently identified (Figure 2). Furthermore, the increase in XDR TB during 2006–2007 suggests ongoing transmission of XDR TB.

Study limitations include unavailability of data to assess trends in culture-taking practices, so we could not evaluate how culture-taking practices may have influenced the increase in reporting of MDR TB over time. Also unavailable were data for categorizing MDR TB cases as new or retreatment.

Our findings show that incidence rates of MDR TB in KwaZulu-Natal could be higher than previously estimated. Since 2007, steps have been taken to validate and implement rapid diagnostic tests for all TB patients in the province and to increase access to MDR TB treatment by increasing bed capacity and decentralizing the MDR TB treatment program. The challenge is ensuring that all patients with a new diagnosis of MDR TB have access to treatment. Similarly, ongoing transmission of TB must be reduced by implementation of sound infection control measures.

Acknowledgments

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Antimicrobial Ointments and Methicillin-Resistant *Staphylococcus aureus* USA300

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We tested 259 methicillin-resistant *Staphylococcus aureus* isolates and 2 USA300 ATCC type strains for susceptibility to bacitracin and neomycin contained in over-the-counter antibacterial ointments. Resistance to both bacitracin and neomycin was found only in USA300. The use of over-the counter antimicrobial drugs may select for the USA300 clone.

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is rapidly spreading worldwide. MRSA USA300 is a clone of increasing public health concern among rapidly disseminating CA-MRSA strains in the United States (*1*). MRSA USA300 is designated as sequence type (ST) 8 by multilocus sequence typing (MLST) and possesses staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa. Although the rapid dissemination of the USA300 clone may occur because of a high virulence level that arises from the production of Panton-Valentine leukocidin (PVL) or an existing arginine catabolic mobile element (ACME) (*2*), there is no conclusive evidence to support this hypothesis (*3*). Furthermore, the hypothesis cannot account for the rapid dissemination of MRSA in countries where USA300 clones are not the dominant clones (most European countries, South Korea, and Japan) (*4*–*6*). In most European countries, the dominant CA-MRSA clone is the European clone (ST80, SCC*mec* type IV, PVL positive and ACME negative) (*1*,*4*). In South Korea, only 1 isolate was a USA300 clone among 138 MRSA isolates collected from patients with bacteremia and soft tissue infection (*5*). In Japan, the MRSA USA300 clone is rare (*6*).

In many cases, soft tissue infection acquired in communities was treated by using over–the-counter (OTC) drugs called triple-antibiotic ointment (TAO), e.g., Neosporin (polymyxin B [PL-B] sulfate, 5,000 units/g; bacitracin, 400 units/g; and neomycin, 3.5 mg/g) and Polysporin triple ointment (PL-B sulfate, 10,000 units/g; bacitracin, 500 units/g; and gramicidin 0.25 mg/g). These ointments contain antimicrobial drugs at concentrations far exceeding their MICs among *S. aureus* strains (16–32 μg/mL [equivalent to 124–248 unit/mL] for PL-B, <1–64 units/mL for bacitracin, and <1–>128 μg/mL for neomycin) (*7*,*8*). It is hypothesized that CA-MRSA cases in the United States were under the selective pressure of TAOs.

In this study, we tested the susceptibilities of MRSA isolates, including the USA300 clone, to the antimicrobial drugs in TAOs. We also considered the possible role of TAOs in spreading the USA300 clone.

The Study

We selected 222 MRSA isolates that were not classified as the New York/Japan (NY/JP) clone on the basis of the absence of SCC*mec kdpC* (*9*). In addition, 37 NY/JP clone-like isolates were used. A total of 259 MRSA isolates were tested in our study. Of these 259 isolates, 227 were collected during 2004–2010 at Nagoya Medical Center, and 32 isolates were collected in 2006–2009 at Kyoto University Hospital, including 9 USA300 outbreak isolates (*6*). Details of isolates used in this study are shown in Table 1. ATCC BAA1556 (USA300 FPR3757) (American Type Culture Collection, Manassas, VA, USA) and ATCC BAA1717 (USA300-HOU-MR TCH1516) strains were also used in our study. Susceptibilities to bacitracin and neomycin were tested by the Kirby-Bauer disk diffusion method (Becton Dickinson, Franklin Lakes, NJ, USA). MICs of bacitracin, neomycin, and PL-B for USA300 strains were determined by the agar dilution method according to the Clinical and Laboratory Standard Institute M07-A8 guidelines (*10*). To observe interaction among these 3 antimicrobial drugs, a double-disk synergy test was performed with modification by using ATCC BAA1717 (*11*).

SCC*mec* were determined according to the method of Hisata et al. (*12*). Isolates possessing both PVL and *arcA* (*13*) were analyzed by pulsed-field gel electrophoresis as described in our previous study (*9*). Moreover, USA300 isolates were genotyped by MLST (www.mlst.net) and staphylococcal protein A (*spa*) typing (www.spaserver.ridom.de).

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Table 1. Source of methicillin-resistant *Staphylococcus aureus* isolates, Japan, 2004–2010

Source	Outpatients		Inpatients		Health care workers
	No. community-acquired infections*	No. hospital-acquired infections	No. community-acquired infections*	No. hospital-acquired infections	
Skin and soft tissue	23	7	4	23	0
Bloodstream	0	2	1	19	0
Respiratory tract	0	2	1	17	0
Urinary tract	0	2	2	3	0
Ear	8	0	0	1	0
Eye	3	0	0	3	0
Others	1	1	2	3	0
Carriage	14	10	3	29	0
Screening	50	0	2	20	3
Total	99	24	15	118	3

*Community-acquired infections were determined on the basis of patients' histories according to Centers for Disease Control and Prevention (Atlanta, GA, USA) guidelines (www.cdc.gov/mrsa/diagnosis/index.html).

Nineteen of the 259 isolates harbored both the PVL and the *arcA* gene. Of these 19 isolates, 18 had been collected from Kyoto University Hospital and 1 from Nagoya Medical Center (Table 2). All 19 PVL- and ACME-positive isolates were determined to be ST8 by MLST. These isolates showed USA300 PFGE patterns identical to ATCC BAA1556 and were of SCC*mec* type IVa. SCC*mec* elements of other isolates were determined as type I (n = 4), IIa (n = 37), IIb (n = 52), II untypeable (n = 14), IV (n = 104), and V (n = 9). The SCC*mec* element of the remaining 20 isolates could not be identified.

The 18 USA300 isolates collected from Kyoto University Hospital showed the same *spa* type (t008). However, the 1 USA300 isolate collected from Nagoya Medical Center was of *spa* t190.

ATCC BAA1717 and 9 USA300 isolates collected during 2007–2009 at Kyoto University Hospital were resistant to both bacitracin and neomycin. The USA300 isolate detected at Nagoya Medical Center in 2004 was bacitracin resistant and neomycin susceptible. The other 9 USA300 isolates and ATCC BAA1556 were susceptible to both drugs (Table 2). Highlander et al. (*14*) found that the bacitracin- and aminoglycoside-resistant genes were located on pUSA300-HOU-MR, a plasmid typically observed in the USA300 strain TCH1516. The resistance to bacitracin and neomycin may depend on the presence of the plasmid and may be absent in some USA300 clones.

On the other hand, nearly all MRSA isolates that were determined to be a type other than USA300 were susceptible to bacitracin. One isolate was determined to have intermediate resistance to bacitracin. Also, 11 (4.5%) of the 240 MRSA isolates not deemed to be USA300 were resistant to neomycin, while 132 (55%) demonstrated intermediate resistance (Table 2). A study performed in the 1990s reported that most MRSA strains were susceptible to bacitracin, and many were resistant to neomycin (*8*). Our findings were consistent with the previous study.

MICs of bacitracin, neomycin, and PL-B were 400 units/mL, 128 μg/mL, and 400 units/mL, respectively, among most USA300 isolates with resistance to both bacitracin and neomycin (Table 2). The concentrations of neomycin and PL-B in the TAOs were ≈10 to 30× higher than the MICs of both drugs. In addition, neomycin and PL-B were observed to be weakly synergistic (Figure). However, Bearden et al. reported that despite containing antimicrobial drugs at concentrations far exceeding their MICs among MRSA, PL-B and neomycin ointment, or PL-B and gramicidin ointment exhibited deficient bactericidal activity in time-kill assays (*15*). Bacitracin may thus be required for sufficient bactericidal activity. Acquiring resistance to bacitracin and neomycin may be essential for survival under the selective pressure of TAOs. If so, bacitracin resistance should be considered a key characteristic of the USA300 clone.

Table 2. Bacitracin and neomycin susceptibility of MRSA USA300 and other MRSA isolates*

Bacitracin/neomycin	MRSA USA300 (MICs of bacitracin, neomycin, and polymyxin B)†			Other MRSA
	Kyoto University Hospital	Nagoya Medical Center	ATCC type strains	
R/R	9 (400, 128, 200–400)	0	BAA1717 (400, 128, 400)	0
R/S	0	1 (400, 0.25, 400)		0
S/R	0	0		11
I/S	0	0		1
S/I	0	0		132
S/S	9 (6.25–12.5, 0.25, 400)	0	BAA1556 (6.25, 0.25, 400)	96
Total	18	1	2	240

*MRSA, methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection (Manassas, VA, USA); R, resistant; S, susceptible; I, intermediate resistance.
†MICs are expressed as units/mL for bacitracin and polymyxin B and in μg/mL for neomycin.

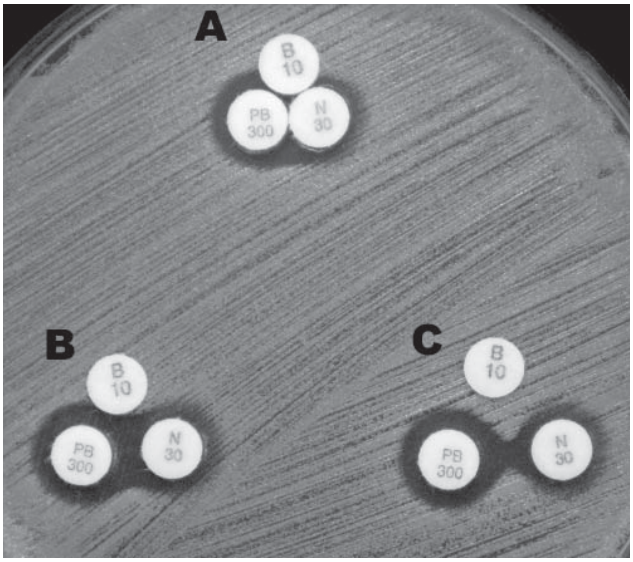

Figure. Double-disk synergy test with 3 disks, bacitracin (B10 disk), neomycin (N30 disk), or polymyxin B (PL-B, PB300 disk) was performed with USA300 strain ATCC BAA1717. Disks were placed at 6 mm (A), 9 mm (B), and 11 mm (C) distance from disk centers. Neomycin and PL-B were found to be weakly synergistic.

TAOs containing bacitracin, neomycin, and PL-B are widely used in the United States; thus, bacitracin- and neomycin-resistant strains may be selected by the selective pressure of the TAOs. Although bacitracin and neomycin ointments are also available as OTC drugs in Japan, use of the ointments is not widespread. As a result, the selective pressure that leads to bacitracin and neomycin resistance is weak in Japan.

Conclusions

The emergence of MRSA USA300 depends partly on the virulence of MRSA USA300, but it may be influenced by usage of OTC drugs. In each country, susceptibilities of MRSA USA300 to bacitracin and neomycin should be thoroughly investigated, and relationships between the dissemination of MRSA USA300 and the usage of OTC drugs should be clarified. Such an investigation will provide valuable information regarding the emergence of organisms resistant to OTC topical antibiotics and likely a warning against the indiscriminate use of antimicrobial drugs. Further studies are required to validate these findings.

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Novel Arenavirus, Zambia

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To investigate arenavirus in Zambia, we characterized virus from the kidneys of 5 arenavirus RNA–positive rodents (*Mastomys natalensis*) among 263 captured. Full-genome sequences of the viruses suggested that they were new strains similar to Lassa virus–related arenaviruses. Analyzing samples from additional rodents and other species can elucidate epizootiologic aspects of arenaviruses.

Arenavirus, a bisegmented ambisense single-stranded RNA virus, is 1 of the viral pathogens responsible for hemorrhagic fever in Africa and South America. Until 2007, Lassa virus was the only known arenavirus to cause hemorrhagic fever in Africa. However, during September–October 2008, 5 hemorrhagic fever cases caused by a novel arenavirus named Lujo virus occurred in South Africa (*1*). The initial case occurred in Zambia; the patient was transported to South Africa for treatment, where the virus spread to 4 other persons. Four patients died; the source of infection in the index patient was not determined.

The natural reservoir of arenavirus in Africa is rodents of the family Muridae, especially *Mastomys natalensis*, and nonpathogenic arenaviruses have been found in areas surrounding Zambia (*2*–*5*). To further the epizootiologic understanding of arenaviruses, we investigated their prevalence and genetic background among *M. natalensis* rodents in Zambia during May 22–August 28, 2009.

The Study

We conducted the study with permission from the Zambia Wildlife Authority. Sherman traps (H.B. Sherman, Inc., Tallahassee, FL, USA) were set up on dry, arable lands or scrublands surrounding the cities of Lusaka (15°26′30.85″S, 28°26′51.09″E), Namwala (15°43′01.37″S, 26°42′33.41″E), and Mfuwe (13°6′47.92″S, 31°48′17.24″E). We captured 57, 48, and 158 rodents in each of these cities, respectively. Rodents were euthanized with diethyl ether, and kidney tissues were harvested and stored at −80°C.

For species identification of the rodents, DNA was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN, Chuo-ku, Tokyo). *Mastomys* spp. were identified by mitochondrial cytochrome b gene (*cytb*) (*6*). Two primers, mCytb-F (5′-ACCCACTGTT TAAAATTATTAACCACTC-3′) and mCytb-R (5′-CTC CGATTCAAGTTAGTACTAGTAG-3′) were used for PCR amplification of *cytb*. BLAST (www.ncbi.nlm.nih.gov/blast.cgi) search analysis against the amplified *cytb* fragments showed that 23 of 57 rodents from Lusaka, 24 of 48 from Namwala, and 143 of 158 from Mfuwe were *M. natalensis*.

The QIAGEN OneStep RT-PCR Kit (QIAGEN) was used to screen arenaviruses under the following conditions: 30 min at 50°C, 15 min at 95°C, 45 cycles of 20 s each at 95°C, 30 s at 50°C, 1 min at 72°C, and 10 at 72°C. The primer sequences used were 5′-CACATAGTTGGGCCCCACTTGCTGTGATC-3′ and 5′-AGGATAAGTGAAAGAGAGAGTAATTC-3′, which were designed on the basis of a consensus sequence of the large (L) gene among African arenavirus strains (Old World Arenaviruses [OWAs]), including Lujo virus. The region of the L gene has been reported as well conserved among OWAs (*7*).

Total RNA samples were extracted from kidney tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) PCR results indicated that 4 (17%) of the 23 rodents captured in Lusaka and 1 (4%) of the 24 captured in Namwala were positive for arenavirus, but none of the 143 rodents captured in Mfuwe were positive. Overall, 5 (3%) of the 190 *M. natalensis* rodents trapped in Zambia were positive for arenavirus. All amplicons were confirmed by nucleotide sequencing and analyzed by BLAST search.

We used RNA samples extracted from kidney tissues of representative Lusaka and Namwala strains to determine full-genome sequences. After several attempts to amplify the virus genome cDNA of OWAs by using deduced universal primers, we obtained some virus fragments and determined the full-genome sequence by the gap closing and rapid amplification of cDNA ends methods. GenBank accession numbers for the Lusaka strain are AB586644 and AB586645, and for the Namwala strain, AB586646 and AB586647. Sequence analysis indicated that the genome of the Lusaka and Namwala strains have a typical bisegmented structure containing 2 open reading frames in each segment, and the genes in the segments are separated by a stable stem-loop structure (data not shown). The small segments of the Lusaka and Namwala strains are both 3,377 bp, and the large segments are 7,230 and 7,236 bp, respectively. The shortage in the Lusaka strain genome,

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Table 1. Nucleotide sequence comparison among Old World arenaviruses*

Strain	Lusaka	Namwala	MOBV	MORV	MOPV	LASV	LUJV
Lusaka	0						
Namwala	0.130	0					
MOBV	0.365	0.367	0				
MORV	0.372	0.365	0.392	0			
MOPV	0.375	0.385	0.388	0.279	0		
LASV	0.406	0.406	0.413	0.434	0.417	0	
LUJV	0.640	0.646	0.667	0.670	0.654	0.666	0

*MOBV, Mobala virus (GenBank accession no. NC_007903); MORV, Morogoro virus (NC_013057); MOPV, Mopeia virus (NC_006575); LASV, Lassa virus (NC_004296); LUJV, Lujo virus (FJ952384). The number of nucleotide substitutions per site is shown. The small segment nucleotide sequences from 7 arenavirus strains were analyzed. All positions containing gaps and missing data were eliminated. The final dataset comprised 3,130 positions.

compared with the Namwala strain genome, was in a noncoding region between the stem-loop and the L gene.

We used MEGA5 software (*8*) to calculate values of diversity between the genomic small segment of the Lusaka strain and several OWAs. The lowest value, 0.13, was for Namwala strain; values ranged from 0.365 to 0.640 for the OWAs (Table 1). The divergence among each virus strain, except for the Zambian strains, ranged from 0.279 to 0.670 (Table 1). Analysis of the large segments showed similar results (data not shown). Phylogenetic analysis based on the deduced amino acid sequences of the 4 virus proteins showed clearly distinct sequences between OWAs and South American strains (New World arenavirus) (Figure 1, panels A–D; OWAs indicated in the gray

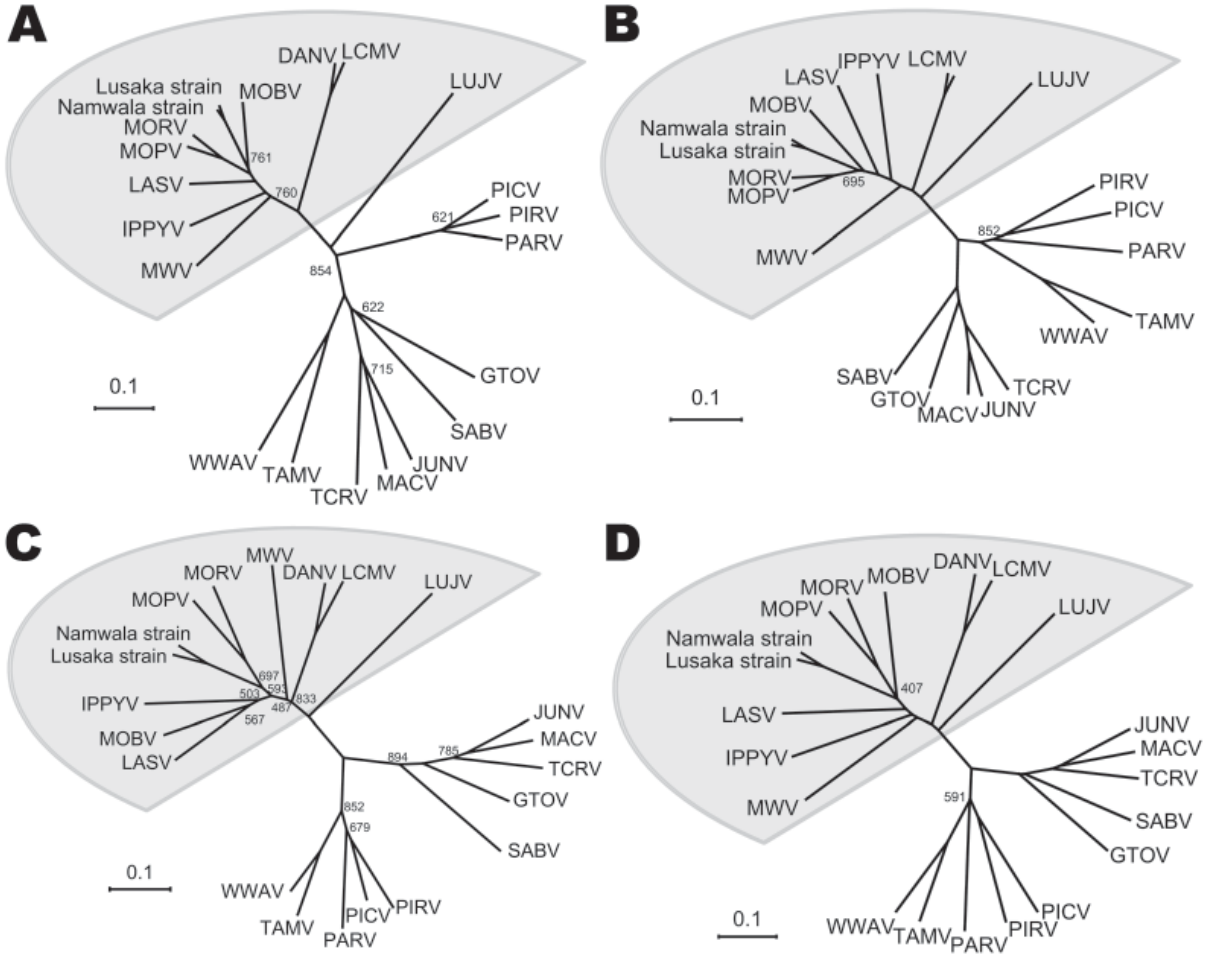

Figure 1. Phylogenetic analysis of Luna virus proteins based on the amino acid sequence, Zambia, 2009. Phylogenetic tree of A) glycoprotein precursor, B) nucleoprotein (NP), C) Z protein, and D) L protein. Bootstrap values are indicated in the trees (<900). Data from which amino acid sequences used for phylogenetic analyses were deduced are provided online (www.cdc.gov/EID/content/17/10/10-1452-F1.htm). Scales bars indicate amino acid substitutions per site.

Table 2. Functional motifs in the Z and glycoprotein precursor proteins of Old World arenaviruses*

Strain	Late domains†	Strain	S1P cleavage site‡
LUNV (Lusaka)	**PTAP**KESASN**PPPY**SP	LUNV (Lusaka)	RRLM-GTFTW
LUNV (Namwala)	**PTAP**KEPARN**PPPY**SP	LUNV (Namwala)	RRLM-GTFTW
MOBV	**PTAP**PPEATN**PPPY**SP	MOBV	RRLM-GTFTW
MOPV	**PTAP**PEIPPSQN**PPPY**SP	IPPYV	RRLM-STFTW
MORV	**PTAP**PEAMPSQQ**PPPY**QP	LUJV	RKLM-KLFQW
LASV	**PTAP**PTGAADSIR**PPPY**SP	MOPV	RRLL-GLFTW
IPPYV	**PSAP**PSPS**PPPY**SP	MORV	RRLL-GLFTW
LCMV	**STAP**SS**PPPY**EE§	LASV	RRLL-GTFTW
DANV	**STAP**SS**PPPY**EE	LCMV	RRLA-GTFTW
LUJV	**PSAP**PL	DANV	RRLA-GTFTW

*S1P, cellular proprotein convertase site 1 protease; LUNV, Luna virus; -, S1P cleavage site; MOBV, Mobala virus; MOPV, Mopeia virus; MORV, Morogoro virus; LASV, Lassa virus; IPPYV, Ippy virus; LCMV, lymphocytic choriomeningitis virus; DANV, Dandenong virus; LUJV, Lujo virus.
†Conserved P(T/S)AP and PPPY motifs in the Z protein are in **boldface**.
‡R(R/K)LM is considered a putative, although unconfirmed, S1P cleavage site.
§STAP is considered a putative, although unconfirmed, PTAP motif.

area). The Lusaka and Namwala strains were classified as members of the OWAs; both strains are closely related to the Mobala, Morogoro, and Mopeia viruses. Thus, we concluded that the Zambian strains belong to the same virus species and that the novel arenavirus differs from other known strains. We propose that these Zambian strains be designated Luna virus (Lusaka-Namwala).

The characteristic functional motifs of the Z and glycoprotein precursor (GP-C) were well conserved in Luna virus. The Z protein has a critical role in arenavirus budding, and 2 conserved late-domain motifs, P(T/S)AP and PPPY, in the C-terminal have been reported (*9*,*10*). Luna virus also exhibited the P_{83}TAP and P_{93}PPY motifs, which are present in other OWAs, excluding Lujo, Dandenong, and lymphocytic choriomeningitis viruses (Table 2). GP-C was posttranscriptionally processed by S1P (the cellular proprotein convertase site 1 protease) to yield the glycoproteins, and the consensus motif R-(R/K/H)-L-(A/L/S/T/F) was identified as the S1P recognition site of Luna virus glycoprotein (*11*,*12*). Luna virus GP-C contained R_{257}RLM, which is apparently cleaved in a similar fashion; however, its cleavage mechanism has not yet been confirmed. The RRLM sequence is also conserved in the Mobala and Ippy viruses (Table 2). The details of this protein motif suggested that Luna virus is more similar to Mobala virus than to Mopeia and Morogoro viruses.

We attempted to isolate Luna virus from the 5 viral RNA–positive tissue samples. Each kidney homogenate was injected into Vero E6 cells in Dulbecco modified Eagle medium supplemented with 2% fetal bovine serum. The culture medium was changed every 6 days, and the supernatant was harvested after 28 days of cultivation. The harvested culture supernatant was injected into new Vero E6 cells. During the cultivation period, culture supernatant was sampled every 2 days and tested for the presence of Luna virus RNA by 1-step RT-PCR. Finally, the amount of viral RNA in the culture supernatant of 1 Lusaka sample was increased during days 6–12 (Figure 2). During this time, distinct cytopathic effect was not observed (data not shown). To observe the virus particles, the culture supernatant was ultracentrifuged at 100,000 × *g*, and the precipitates were negatively stained with 2% phosphotungstate. Transmission electron microscopy indicated typical round-shaped, enveloped particles, 75 nm in diameter, with electron-dense dots inside the envelope (data not shown).

Conclusions

We isolated a novel nonpathogenic arenavirus, which we propose be designated Luna virus, from *M. natalensis*

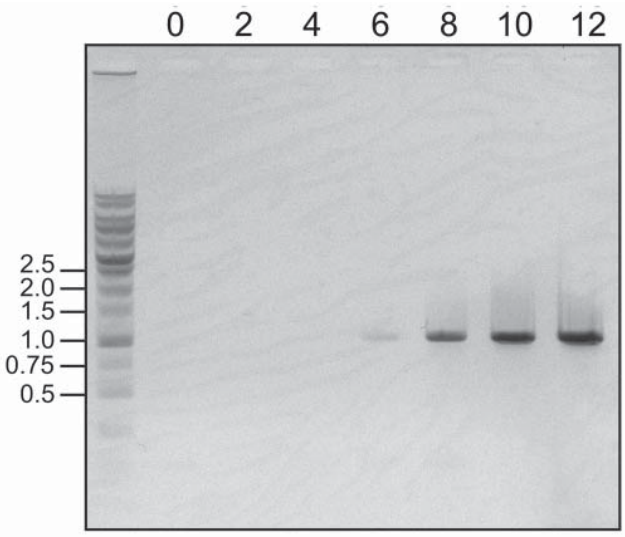

Figure 2. Detection of increasing viral RNA by 1-step reverse transcription PCR in a novel arenavirus, Zambia, 2009. Viral RNA was extracted from 100 μL of culture supernatant on the indicated days (top) and eluted in 20 μL of distilled water. The RNA sample was subjected to 1-step reverse transcription PCR with the specific primers 5′-TGAGAGACATTGCTTCACAATTGACATCC-3′ and 5′-TGACCCATTCTTGATGTATTGTGACTCC-3′, which were designed to amplify a 1,000-bp fragment within the determined large gene segment of Luna virus. DNA size markers are shown in the far left lane; sizes in kb are indicated at left.

rodents in Zambia. Comparison of the genetic backgrounds of Luna virus and Lujo virus, a novel pathogenic arenavirus also found in Zambia, showed that Luna virus is genetically different from Lujo virus. Luna virus was closely related to nonpathogenic arenaviruses that have been found from central to eastern Africa. To elucidate the epizootiologic aspects of arenaviruses in Zambia, the number of rodent and other species samples must be expanded. Such elucidation can lead to discovery of new arenaviruses, as demonstrated by isolation of a pathogenic New World arenavirus from bats during a study to increase knowledge of the geographic range and genetic diversity of arenaviruses naturally associated with the Mexican woodrat (*Neotoma* mexicana) in the western United States (*13*).

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Pandemic (H1N1) 2009 Encephalitis in Woman, Taiwan

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We report an unusual case of pandemic (H1N1) 2009–related encephalitis in an immunocompetent woman. Although rare cases of adult pandemic (H1N1) 2009 associated with encephalitis have been reported previously, in this patient, direct viral invasion of the central nervous system was shown by simultaneous detection of viral RNA and pleocytosis.

Neurologic complications of pandemic (H1N1) 2009 were first reported in children in May 2009 (*1*). Most subsequent reports were also of cases in children, because those <17 years of age appear most vulnerable (*2*). To our knowledge, only 1 retrospective review has documented the frequency of neurologic dysfunction among adults with pandemic (H1N1) 2009 (*3*). In this Asian cohort of 826 hospitalized adults, seizures developed in 6 of the 9 persons with neurologic manifestations, and none had encephalopathy or encephalitis. Thus, seizures appear to be the most common reason for seeking care for children and adults, followed by encephalopathy, in particular, acute necrotizing encephalopathy in children (*4*). We performed a Medline search of literature in English and found 5 case reports of adults with pandemic (H1N1) 2009 encephalitis. Here, we report a woman who sought care because of focal neurologic deficit.

The Patient

The patient was a previously healthy 60-year-old housewife of Mandarin Chinese descent with well-controlled essential hypertension. Her regular medications consisted of once-daily doses of bisoprolol, irbesartan-chlorothiazide, and lercanidipine. She lived in a bungalow in New Taipei City, Taiwan, with her daughter and husband, who both remained asymptomatic. No travel, animal or insect contact, or influenza vaccination history was elicited.

Sore throat, epiphora, and otalgia developed 3 days before hospital admission to Far Eastern Memorial Hospital, New Taipei City. High sustained fever (up to 40°C) and abrupt left lower face paresthesias prompted a clinic visit the following day. Her family physician performed a rapid influenza diagnostic test, which had negative results. Nevertheless, she received empirical oseltamivir, according to the Taiwan Department of Health's emergent initiatives for the peak pandemic (H1N1) 2009 season. After 3 doses of oseltamivir, she remained febrile, with progressive anorexia, malaise, and dizziness, and sought care at the emergency department of a medical center.

At the emergency department (day 4 of symptoms), acute urinary retention developed in the patient without anal sphincter involvement or saddle paresthesias. Despite sensing urgency of her full bladder, she was unable to void spontaneously, necessitating catheterization. She exhibited no other neurologic deficits, seizures, altered mentation, or meningism. Her respiratory symptoms remained mild and confined to the upper respiratory tract. Myalgia, arthralgia, and gastrointestinal symptoms were not prominent.

On admission, she had a temperature of 38.5°C but appeared well. Her blood pressure, pulse, and respiratory rate were 125/64 mm Hg, 89 beats per minute, and 20 breaths per minute, respectively. Results of her physical examination were unremarkable except for the relative bradycardia (which may have been attributable to β-blocker use) and an above average body mass index of 28 kg/m^2. A neurologic examination found diminished sensation to light touch over the distribution of her left trigeminal mandibular nerve. Her other cranial and peripheral nerve functions and higher cortical functions were grossly intact. Ear, nose, and throat examination revealed the normal appearance of bilateral eardrums and pharynx.

Initial hemogram showed borderline leukocytosis (10,340 cells/μL) with relative lymphopenia (17% lymphocytes) and a platelet count within reference range (210 × 10^3/μL). Biochemical testing revealed renal function, electrolytes, serum alkaline phosphatase, and transaminase levels within reference ranges. Plain chest radiographs and results of urinalysis were unremarkable. Electroencephalogram showed symmetric basal activities without epileptiform discharges. Magnetic resonance imaging (MRI) of the brain on day 9 of symptoms showed multifocal scattered T2 high-signal lesions over bilateral hemispheres, occipital horn, basal ganglion, and brainstem, involving both gray and white matter, with diffusion restriction (Figure, panel A). The ventriculitis correlated with her acute urinary retention.

Cerebrospinal fluid (CSF) analysis was performed on days 8 and 11 (Table). Transition from a neutrophilic to a lymphocytic predominant picture was observed. CSF cryptococcal antigen test; Gram, India ink, and acid-fast stains; and subsequent bacterial, mycobacterial, and fungal cultures all yielded negative results. By real-time PCR screening of a wide panel of possible infectious etiologic

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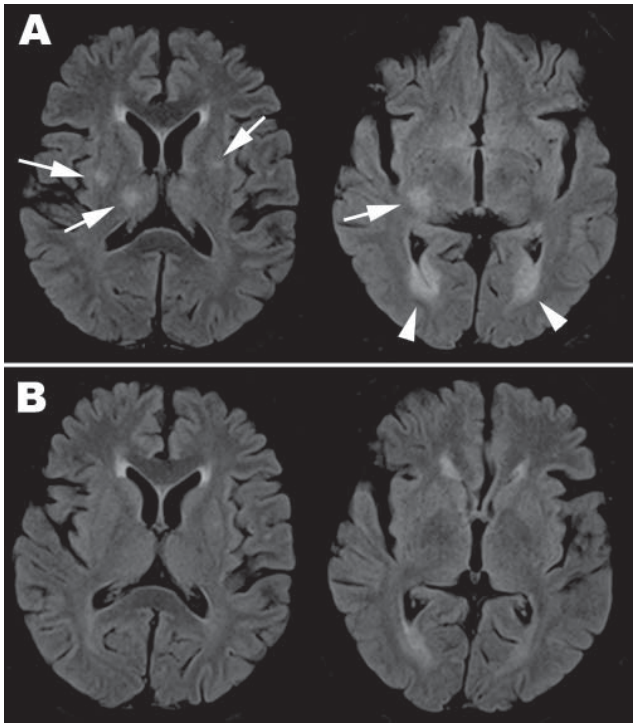

Figure. Magnetic resonance imaging with fluid-attenuated inversion recovery sequence of brain for adult patient with pandemic (H1N1) 2009 encephalitis, Taiwan. A) On day 9 after symptom onset, scattered asymmetric focal hyper signal intensities over bilateral putamen and right thalamus (arrow on the left image) and ventriculitis over bilateral occipital horns (arrow head over right image) are seen. B) By day 24, the lesions had resolved.

agents, influenza A (nonsubtyped) was simultaneously identified in CSF, nasopharyngeal swab specimen, and blood on day 11.

A regimen of oseltamivir (75 mg 2×/d), initiated at onset of fever, was completed after 5 days. Defervescence occurred slowly over 2 weeks. Urodynamic study revealed detrusor spasticity, which was successfully treated with a combination of diazepam and phenazopyridine. Repeat brain MRI on day 24 showed near total resolution of the T2-hyperintense lesions (Figure, panel B). However, after 2 months, the patient reported residual left facial numbness.

Conclusions

According to the Centers for Disease Control in Taiwan, the circulating influenza A strain for the winter-spring season of 2011 was pandemic (H1N1) 2009 with few exceptions (*5*). The pandemic signature of novel subtype H1N1 has been its predilection for infecting healthy adults and its high transmissibility; hence, we were unable to trace contact history in the case described here. The patient did not have known comorbid risk factors; hence, her clinical course was mild. Epidemiologic clues to her diagnosis included residence in an area with the highest incidence of pandemic (H1N1) 2009 in Taiwan in 2011 and her naive immunity (lacking vaccination or exposure to the pandemic 1918 strain). Clinical clues supporting pandemic (H1N1) 2009 infection include initial leukocytosis as opposed to leukopenia, relative lymphopenia, and initial false-negative rapid influenza diagnostic test (*6*).

Only a few cases of pandemic (H1N1) 2009 encephalitis in adults have ever been reported (online Appendix Table, www.cdc.gov/EID/content/17/10/11-0916-appT.htm). All patients had altered mental status with or without seizures. The onset of neurologic symptoms usually occurs within a few days of influenza-like illness. Unlike children, for whom the mortality rate can be as high as 30% (*4*), most adults survive, despite varying degrees of sequelae. Initial neurologic severity parallels the severity of pulmonary disease and is predictive of neurologic outcomes. This case highlights the possibility that subtle neurologic deficits may lead to underrecognition of the milder spectrum of central nervous system (CNS) complications associated with influenza. The patient's report of focal paresthesias and micturition difficulties (in the absence of global neurocognitive defects) understates the substantial, albeit transient, CNS inflammation captured on serial CSF and MRI studies. MRI patterns of CNS inflammation in adults appear nonspecific, with T2 lesions distributed across both white and gray matter, with or without symmetry, brain necrosis, infarct, hemorrhage, edema, or ventriculitis.

This patient may be the eldest and only female adult reported with pandemic (H1N1) 2009 encephalitis. As observed, other adults were men from 20 to 40 years of age (online Appendix Table). There may be a yet unidentified genetic predisposition for influenza-related encephalopathy to develop among Asians, as noted previously in children (*4*) (and possibly among male adults). A remarkable feature of our case was the simultaneous CNS detection of virus and pleocytosis, which suggests that the pathogenesis of subtype H1N1 encephalitis may not be simply due to immune activation or cytokine storm as current favored hypotheses propose but also may be caused by direct viral invasion. One possible mechanism is that the virus crosses the blood–brain barrier by way of the peripheral nerves (*12*). Although we cannot confirm the entry portal of the

Table. Sequential cerebrospinal fluid analysis for adult patient with pandemic (H1N1) 2009 encephalitis, Taiwan*

Cerebrospinal fluid	Day 8	Day 11	Reference range
Protein	94	78	15–40 mg/dL
Glucose	61	61	40–70 mg/dL
Lactate	2.9	2.7	1–2 mmol/L
Leukocytes (Lymph:PMN)	244 (1:99)	64 (98:2)	0–5 L/mm^3
Erythrocytes	12	22	0/mm^3

*Lymph:PMN, lymphocytes:polymorphonuclear cells.

virus, we note that when the patient sought care, she had a peripheral cranial nerve deficit. Influenza A virus has been detected in the CSF of a small minority of Japanese children (especially in those with severe brain pathology) (*13*) and in 1 teenager in South Korea (*14*). In children, pleocytosis has rarely been described, whereas in this adult series, mild pleocytosis appears not infrequently (online Appendix Table). Despite the 2008 recommendation of the Infectious Diseases Society of America regarding routine lumbar puncture in the management of encephalitis for survey of possible etiologic agents (*15*), CSF reverse transcription PCR for influenza is infrequently performed in adults (online Appendix Table). Therefore, the role of viral CNS invasion may be underestimated.

In conclusion, physicians (not just pediatricians) should be alert to the possibility of neurologic disease due to pandemic (H1N1) 2009, especially in persons whose symptoms are subtle. Further studies are warranted to clarify and confirm the neurotropism, particularly for persons of Asian heritage, of pandemic (H1N1) 2009.

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Household Transmission of Pandemic (H1N1) 2009 Virus, Taiwan

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During August–November 2009, to investigate disease transmission within households in Taiwan, we recruited 87 pandemic (H1N1) 2009 patients and their household members. Overall, pandemic (H1N1) 2009 virus was transmitted to 60 (27%) of 223 household contacts. Transmission was 4× higher to children than to adults (61% vs. 15%; p<0.001).

Pandemic (H1N1) 2009 was first identified in 2 southern California counties in April 2009 (*1*), and the World Health Organization declared a global pandemic on June 11, 2009 (*2*). In Taiwan, the government suggested that persons with pandemic (H1N1) 2009 remain home until 24 hours after they were symptom free (*3*). In some influenza epidemics, ≈50% of households have ≥1 members who become infected (*4*). Further investigation into the transmission of pandemic (H1N1) 2009 virus among household members is needed to help control and prevent additional infections. We investigated the transmission of pandemic (H1N1) 2009 virus and clinical outcomes of infection within households of persons with laboratory-confirmed infection.

The Study

During August–November 2009, we enrolled patients at the National Taiwan University Hospital who were infected with pandemic (H1N1) 2009 virus and their household members. The following samples were obtained from patients with clinical signs and symptoms suggestive of pandemic (H1N1) 2009 infection who visited the emergency department, outpatient clinics, or inpatient wards: nasopharyngeal swab specimen for rapid influenza antigen testing (QuickVue A+B test; Quidel, San Diego, CA, USA), throat swab specimen for virus isolation and novel subtype H1N1 reverse transcription PCR (RT-PCR), and blood specimen for serum hemagglutination inhibition (HI) assays. Laboratory-confirmed pandemic (H1N1) 2009 infection was defined in 3 ways: 1) isolation of influenza A virus, followed by positive RT-PCR result for pandemic (H1N1) 2009 virus; 2) positive rapid influenza A test result, followed by positive RT-PCR result for pandemic (H1N1) 2009 virus; or 3) pandemic (H1N1) 2009 virus HI titer ≥40. None of the participants had received an influenza subtype H1N1 vaccine before this study.

Persons with laboratory-confirmed pandemic (H1N1) 2009 and their household members were sent a letter and/or received a telephone call inviting them to participate (Figure). After they accepted the invitation, we collected their case report forms, which contained data regarding the source of infection, final diagnosis, clinical manifestations, and course of the disease. The index patient was defined as the first person in a household to have laboratory-confirmed pandemic (H1N1) 2009 (body temperature >38.0°C and/or cough and/or sore throat). All enrolled index patients and their household members provided blood samples for further HI assays. A mean of 45 days (SD 26, median 36, range 12–107 days) elapsed between the first day of illness in the index patient and household investigations, including blood sampling. The household transmission

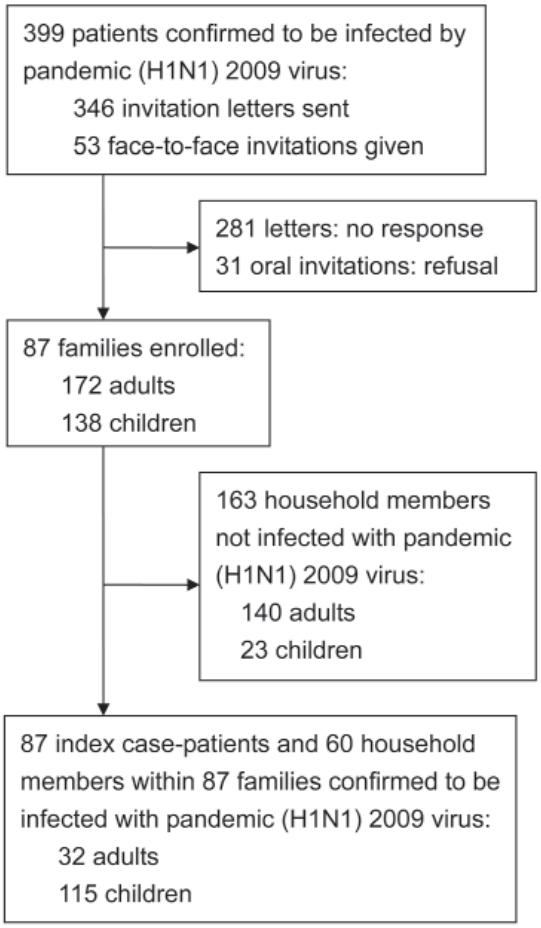

Figure. Flowchart showing household transmission of pandemic (H1N1) 2009 virus infection, Taiwan, August–November 2009.

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rate (secondary attack rate) was defined as the percentage of household members who had laboratory-confirmed pandemic (H1N1) 2009 infection 1–7 days after the onset of symptoms in the index patient.

During August–November 2009, pandemic (H1N1) 2009 was confirmed for 399 patients at National Taiwan University Hospital. Of those 399 patients, 87 patients and their households were enrolled in the study; households included the 87 index patients and their 223 household contacts (172 adults and 138 children) (Figure). Of the 87 index patients, 72 (83%) had visited the hospital for illness and had laboratory-confirmed pandemic (H1N1) 2009 infection (PCR-positive test results, HI titer ≥40, or both); the remaining 15 (17%) index patients attended community clinics and were identified by having titers ≥40 for pandemic (H1N1) 2009 virus on HI test when household investigations were done. The possible source of infection was traced for 52 (60%) of the 87 households: 46 (53%) infections were traced to schools, 5 (6%) infections were traced to daycare centers or other child care situation (when 1 babysitter cared for a few children), and 1 (1%) infection was traced to a summer camp.

As shown in Table 1, the mean ages of the index patients and their household contacts were 10.6 and 33.8 years, respectively; only 6 (7%) of the 87 index patients were adults. Households contained a mean of 1.9 children (SD 0.8, median 2, range 0–4 children).

Pandemic (H1N1) 2009 virus was transmitted to 60 (27%) of the 223 household contacts. The virus was transmitted to 35 (63%) of 56 child-aged siblings (but not to 1 cousin), to none of 5 adult-aged siblings, to 20 (14%) of 138 parents, to 4 (22%) of 18 grandparents, and to 1 (20%) of 5 aunts and uncles. Percentage of transmission among the different groups of household contacts differed significantly: the virus was transmitted to 35 (61%) of the 57 children and to 25 (15%) of the 166 adults ($p<0.01$ by χ^2 test). However, percentage of transmission among different adult groups did not differ significantly ($p = 0.86$ by χ^2 test). Mean interval between the onset of illness in the index patient and household members was 3.3 days (SD 2.6, median 3, range 1–6 days).

Of the 147 patients with pandemic (H1N1) 2009, 119 (81%) received a diagnosis of influenza-like illness; 10% received a diagnosis of upper respiratory tract infection; 3% each received a diagnosis of bronchitis, bronchopneumonia, asthma, or acute gastroenteritis; and 2% received a diagnosis of pneumonia. Of the 147 patients (all children), 10 (7%) were hospitalized and discharged without sequelae. Seventy-seven (89%) of the 87 index patients and 29 (48%) of the 60 household members received oseltamivir.

Table 2 shows attack rates and odds ratios for pandemic (H1N1) 2009 virus infection among the 223 household contacts by patient characteristics (sex and age) and signs and symptoms. Age <18 years, fever, cough, sore throat, rhinorrhea, myalgia, and malaise were significantly associated with pandemic (H1N1) 2009 infection, but age <18 years, fever, and cough most significantly predicted the transmission of pandemic (H1N1) 2009 virus in multivariate analysis with a multiple logistic regression model. However, we did not find a significant relationship between index patient characteristics, specific symptoms, lower respiratory tract infection, or the need for hospitalization and the rate of household transmission of pandemic (H1N1) 2009 virus.

Conclusions

We found children to be >4× more susceptible than adults to the secondary transmission of pandemic (H1N1) 2009 virus within households (61% vs. 15%). Furthermore, 93% of our index patients were children, and for ≈60% of them, the source of exposure to the virus was a school or daycare center. Thus, children play major roles in the introduction and spread of influenza within families.

Table 1. Demographic characteristics and pandemic (H1N1) 2009 infection rates among 223 household contacts of 87 index case-patients, by contact type, Taiwan, August–November 2009*

Study participants	No. participants	Mean age, y (SD)	Sex, F/M	Positive for pandemic (H1N1) 2009		No. positive/no. tested (%)
				By PCR, no.	By HI, no./no. tested (%)	
Index case-patients	87	10.6 (7.2)	42/45	71	81/84 (96)	87/87 (100)
Household members	223	33.8 (17.9)	122/101	21	59/222 (27)†	60/223 (27)
Children	57	8.0 (3.6)	28/29	21	34/56 (61)†	35/57 (61)
Siblings	56	7.9 (3.6)	28/28	21	34/55 (62)†	35/56 (63)
Cousin	1	11.5	0/1	0	0/1 (0)	0/1 (0)
Adults	166	43.2 (10.5)	94/72	0	25/166 (15)	25/166 (15)
Siblings	5	20.3 (1.7)	3/2	0	0/5 (0)	0/5 (0)
Parents	138	41.0 (5.6)	76/62	0	20/138 (14)	20/138 (14)
Grandparents	18	66.6 (5.9)	11/7	0	4/18 (22)	4/18 (22)
Uncles/aunts	5	41.5 (1.7)	4/1	0	1/5 (20)	1/5 (20)
Total	310	27.5 (18.9)	169/141	92	140/310 (45)	147/310 (47)

*HI, hemagglutination inhibition.
†One person was confirmed to be infected with pandemic (H1N1) 2009 virus by PCR of a throat swab specimen without testing of a blood sample.

Table 2. Pandemic (H1N1) 2009 attack rates among 223 household contacts of 87 index patients, by patient characteristics and symptoms, Taiwan, August–November 2009*

Characteristic	Attack rate, %	OR (95% CI)†	p value†
Sex			0.21
M, n = 101	23	Reference	
F, n = 122	30	1.48 (0.81–2.70)	
Age, y			<0.0001
>18, n = 166	15	Reference	
≤18, n = 57	61	9.09 (4.55–17.86)	
Signs and symptoms			
Fever			<0.0001
No, n = 163	12	Reference	
Yes, n = 60	68	16.13 (7.87–33.33)	
Cough			<0.0001
No, n = 158	13	Reference	
Yes, n = 65	60	10.42 (5.29–20.83)	
Rhinorrhea			<0.0001
No, n = 176	19	Reference	
Yes, n = 47	55	5.18 (2.60–10.31)	
Sore throat			0.0002
No, n = 176	21	Reference	
Yes, n = 47	49	3.60 (1.83–7.09)	
Vomiting			0.06
No, n = 214	26	Reference	
Yes, n = 9	56	3.60 (0.93–13.90)	
Diarrhea			0.23
No, n = 214	26	Reference	
Yes, n = 9	44	2.30 (0.60–8.85)	
Malaise			0.002
No, n = 200	24	Reference	
Yes, n = 23	57	4.20 (1.73–10.20)	
Myalgia			0.02
No, n = 192	24	Reference	
Yes, n = 31	45	2.61 (1.20–5.71)	

*OR, odds ratio; CI, confidence interval.
†Univariate logistic regression model was used for unadjusted OR (95% CI) and unadjusted p values. If features were significantly different with p<0.05 in univariate logistic regression model, they were further analyzed with multiple logistic regression model for adjusted OR (95% CI) and adjusted p values.

Vaccination and other measures will prevent susceptible children from becoming infected and reduce influenza virus transmission among families and communities.

This study has limitations, however, for example, the potential for nonresponse bias and possible preferential recruitment of families with sick children as index patients. Thus, adults may be relatively underrepresented as index patients in this study. Also, some adults may be less likely to go to the hospital with influenza-like symptoms.

In our study, the secondary attack rate in households was 27%, which is similar to rates in studies by Komiya et al. (26%), Sikora et al. (30.2%), and Looker et al. (33%) but higher than rates in studies by Cauchemez et al. (13%) and Carcione et al. (14.5%) (*5*–*9*). The secondary attack rate found in this study may have been relatively high because, without a vaccine against pandemic (H1N1) 2009, there were more susceptible children in the households and because most index patients were children who may shed virus for a longer period (*10*). Our findings show the key role that children play in introducing and spreading pandemic (H1N1) 2009 virus within households. Public health measures, such as vaccination and community health education, can prevent infections among children and help reduce virus transmission among families and the larger community.

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Group B Streptococcus and HIV Infection in Pregnant Women, Malawi, 2008–2010

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To determine whether an association exists between group B streptococcus carriage and HIV infection, we recruited 1,857 pregnant women (21.7% HIV positive) from Queen Elizabeth Central Hospital, Blantyre, Malawi. Overall, group B streptococcus carriage was 21.2% and did not differ by HIV status. However, carriage was increased among HIV-positive women with higher CD4 counts.

Group B streptococcus (GBS) is the major cause of neonatal meningitis and septicemia in Malawi, a problem that has only recently been recognized (*1*). A similar pattern of disease has emerged from other countries in eastern and southern Africa (*2*–*8*). The geographic distribution of these reports led us to speculate that an association exists between GBS and HIV infection. Carriage of GBS is a prerequisite for the development of early-onset neonatal GBS disease (*9*) and is a convenient endpoint for a cross-sectional study to assess the interaction of GBS and HIV.

The Study

Pregnant women (≥16 years of age) in their third trimester of pregnancy were recruited from the labor ward of Queen Elizabeth Central Hospital, Blantyre (QECH), during October 2008–March 2010. Recruitment was performed by a single study midwife on Sunday–Thursday each week from 7:30 AM to 4:00 PM, when the midwife was not required to provide emergency care. Women were excluded if they were deemed to be too sick by the study midwife. Following the patient's signed informed consent, medical and reproductive history was recorded, low vaginal and rectal swab specimens were obtained, and after appropriate counseling, a blood sample was obtained to either confirm or determine HIV status. CD4 counts were determined for those who were HIV positive. In accordance with Ministry of Health Guidelines, prevention of mother-to-child transmission care (PMTCT) was offered to women who were HIV infected (*10*); PMTCT was adapted as appropriate to the woman's CD4 count. The mothers were not followed after they left the hospital.

Swab specimens were placed into Todd-Hewitt broth (Oxoid Ltd, Basingstoke, UK) supplemented with 15 μg/mL nalidixic acid and 10 μg/mL colistin and then incubated for 18–24 h. GBS were identified by phenotypic characteristics, CAMP test, and serologic analysis (Oxoid). Serotyping of GBS was performed by use of a commercial serotyping kit (Statens Serum Institut, Copenhagen, Denmark). HIV testing was conducted by using a method based on rapid tests (*11*).

The study aimed to recruit 1,950 women to show a GBS carriage prevalence increased by ≥40% in the HIV-positive women at a significance level of 5% and power of 80%. This assumed 20% HIV prevalence in the labor ward attendees (thus a 4:1 ratio of HIV-negative to HIV-positive women) and 15% GBS carriage in HIV-negative women. Two analyses were planned a priori. The primary analysis was to compare GBS carriage prevalence by HIV status; the secondary analysis was the association of GBS carriage by CD4 count in HIV-infected women. The study was approved by the College of Medicine Research and Ethics Committee of the University of Malawi and the Ethics Committee of the London School of Hygiene and Tropical Medicine.

A total of 16,456 women attended QECH labor ward during the study period. Of these women, 11,861 attended on a recruitment day, and 8,099 attended during recruitment hours, of whom 1,857 (23%) were recruited into the study (Table 1). The 2 primary reasons for not enrolling women were that they were in the second stage of labor and that their hospitalization was caused by an emergency or due to a complicated pregnancy (50.8% of all attendees). The remaining nonrecruits were women who could not be assessed by the study midwife within the specified time. During the same period, 14,783 total deliveries took place at QECH, with 380 multiple births (2.6%), 2,962 caesarean sections (20.0%), 514 stillbirths (3.5%), 29 neonatal deaths, and 20 maternal deaths recorded on the labor ward. The percentage of women in the study who had multiple births (2.7%) and caesarean sections (20.6%) was the same as that in the larger group attending the labor ward. However, the percentage with stillbirths (1.6%) was less, which is consistent with the exclusion of women who were attending the ward for complicated pregnancies or emergency medical conditions.

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Table 1. Comparison of variables, including age, CD4 counts, and reproductive and medical history, for HIV-negative and HIV-positive study participants, Malawi, 2008–2010*

Variable	All study participants, N = 1,857†	HIV-positive participants, n = 402/21.7%	HIV-negative participants, n = 1,454/78.3%	p value
Mean age, y (SD)	25.2 (5.9)	26.7 (5.5)	24.8 (5.9)	<0.001
No. (%) recruited during wet season (Nov–Mar)	1,057 (57.0)	234 (58.2)	820 (56.4)	0.57
Reproductive and medical history				
Pregnancies, median no. (range)	1 (0–10)	2 (0–8)	1 (0–10)	<0.001
Live births, median no. (range)	1 (0–10)	1 (0–7)	1 (0–10)	<0.001
Previous neonatal death, no. (%)	45 (2.4)	11 (2.7)	34 (2.3)	0.65
Median time since LMP, days (range)	275 (98–371)	275 (145–371)	276 (98–370)	0.17
Unwell at labor ward attendance, no. (%)	114 (6.1)	33 (8.2)	81 (5.6)	0.05
Membranes ruptured >18 h before enrolled, no. (%)	52 (2.8)	12 (3.0)	40 (2.8)	0.79
Previous HIV test, no. (%)	1,664 (89.7)	368 (91.5)	1,296 (89.1)	0.16
Taking ART, no. (%)	–	83 (20.7)	–	
Median duration of ART, mo (range)		4 (1–144)		
Genital ulcer disease, no. (%)	46 (2.5)	32 (8.0)	14 (1.0)	<0.001
Sexually transmitted disease, no. (%)	34 (1.8)	23 (5.7)	11 (0.8)	<0.001
S-P use during last 4 wk of pregnancy, no. (%)	1,779 (96.0)	379 (94.3)	1,395 (96.9)	0.38
Examination and other findings				
First stage of labor, no. (%)	1,298 (69.9)	279 (69.4)	1,019 (70.1)	0.79
Premature labor before 36 wk, no. (%)	270 (14.5)	71 (17.7)	199 (13.7)	0.04
Registered for PMTCT on labor ward, no. (%)	–	333 (82.8)‡	–	
Mean birth weight (SD), kg	2.90 (0.54)	2.81 (0.58)	2.91 (0.52)	<0.001
Delivery by cesarean section, no. (%)	383 (20.6)	76 (18.9)	307 (21.1)	0.33
Stillbirth, no. (%)	30 (1.6)	13 (3.2)	17 (1.2)	0.004
Multiple birth, no. (%)	50 (2.7)	10 (2.5)	40 (2.8)	0.77
Laboratory tests				
Plasma sample for CD4 count, no.	–	383§	–	
CD4 count, median (range)		370 (20–1595)		
No. failed samples¶	18	5	13	
GBS isolated, no. (%)#	390 (21.2)	77 (19.4)	313 (21.5)	0.4
Serotype				
1a	71 (18.2)	11 (14.3)	60 (19.2)	
1b	24 (6.2)	1 (1.3)	23 (7.4)	
2	40 (10.3)	9 (11.7)	31 (9.9)	
3	152 (39.0)	32 (41.6)	120 (38.3)	
4	1 (0.3)	0	1 (0.3)	
5	93 (23.9)	23 (29.9)	70 (22.4)	
6 (1 sample), 8 (2 samples)	3 (0.8)	0	3 (1.0)	
Untypeable, no (%)	6 (1.5)	1 (1.3)	5 (1.6)	

*LMP, last menstrual period; ART, antiretroviral treatment; –, not applicable; S-P, sulfadoxine-pyrimethamine; PMTCT, prevention of mother-to-child transmission care; GBS, group B streptococcus.
†Form for 1 participant was missing.
‡No. includes only participants confirmed as HIV-positive.
§15 failures, 4 samples not obtained.
¶Contamination of the selective broth in 9 cases and incubator failure in 9 cases.
#Failed samples excluded from denominator to derive percentage.

Of the study participants with HIV, >80% had been tested before attending QECH labor ward, most as a part of the PMTCT process. Only 125 (31%) of those with positive test results had received any formal HIV clinic care. GBS carriage was detected in 21.7% of the HIV-negative women and 19.4% of the HIV-positive women (χ^2 = 0.99, p = 0.32) (Table 2). In the HIV-positive women, a difference in GBS carriage was noted by CD4 level: women with CD4 counts >500 cells/mm^3 were >2× more likely than those with counts <200 cells/mm^3 to be GBS carriers. When adjusted for antiretroviral treatment, the number of children previously borne, and age, this finding persisted (Table 2). Carriage in the HIV-infected group with CD4 counts >500 cells/mm^3 (28.2%) was higher than in the HIV-uninfected women (21.7%), but the difference did not reach significance in an unadjusted comparison (χ^2 = 2.65, df = 1, p = 0.11).

To test for unrecorded cotrimoxazole use as an explanation of the CD4-associated carriage findings (i.e., that sicker women with more advanced disease might be

more likely to take cotrimoxazole), we performed a post-hoc subgroup analysis on women who had reported no previous HIV testing or care before their recent PMTCT test, considering it unlikely that they would be taking cotrimoxazole. In this group of 277 women (13 with missing CD4 counts), the trend for increasing odds of carriage with higher CD4 count persisted. The prevalence of carriage in the CD4 groups of <200, 200–500, and >500 cells/mm^3 was 15.9%, 19.1% (odds ratio [OR] 1.25, 200–500 cells/mm^3 vs. <200 cells/mm^3), and 31.0% (OR 2.37, >500 cells/mm^3 vs. <200 cells/mm^3), respectively (p = 0.03, by χ^2 test for linear trend). This pattern was similar in the 125 participants who reported accessing HIV care, with ORs of 1.6 and 2.8 in the same CD4 group comparisons.

Conclusions

In the primary analysis comparing carriage prevalence by HIV status, no overall difference in GBS carriage by HIV status was detected. The overall carriage frequency of GBS of ≈20% is comparable with those in other reports from Africa and the industrialized world. However, in the subgroup analysis of HIV-positive women, contrary to our expectations, GBS carriage was significantly increased at higher CD4 counts. Unrecorded use of antimicrobial drugs, particularly cotrimoxazole prophylaxis, as a confounder for this association was considered and dismissed as an explanation for these findings.

Antiretroviral treatment was not shown as an independent risk factor for carriage, but the cross-sectional design of this study precludes any firm conclusions. With increasing numbers of HIV-positive women using antiretroviral drugs, the effect of treatment-induced improvements in CD4 count and the potential for increased GBS carriage merit further investigation.

Our results showed a trend toward higher GBS carriage in HIV-infected women with CD4 counts >500 cells/mm^3

Table 2. Associations with GBS carriage among 340 pregnant women with and without HIV infection, Malawi, 2008–2010*

Variable	GBS carriage, no. positive for variable/no. total (%)	OR (95% CI)	Adjusted OR (95% CI)
All study participants	390/1,857 (21.0)	–	–
HIV-negative participants	313/1,441 (21.7)	Reference	Reference
HIV-positive participants	77/397 (19.4)	0.88 (0.66–1.17)	0.84 (0.63–1.12)
Age group, y†			
16–19	68/345 (19.7)	2.29 (0.68–7.76)	1.41 (0.40–4.92)
20–24	122/582 (21.0)	2.48 (0.74–8.28)	1.96 (0.58–6.63)
25–29	113/498 (22.7)	2.74 (0.82–9.18)	2.43 (0.72–8.18)
30–34	60/261 (23.0)	2.79 (0.82–9.48)	2.59 (0.76–8.88)
35–39	24/122 (19.7)	2.29 (0.64–8.15)	2.03 (0.56–7.33)
≥40	3/31 (9.7)	Reference	Reference
First pregnancy	137 (24.0)	1.30 (1.01–1.65)	1.62 (1.19–2.19)
Second or subsequent pregnancy	244 (19.6)	Reference	Reference
Recruited during rainy season	235 (22.4)	1.18 (0.94–1.50)	–
Recruited during nonrainy season	155 (19.6)	Reference	–
Birth weight ≤2.5 kg			
No	307 (21.5)	Reference	–
Yes	71 (19.4)	0.88 (0.65–1.17)	–
Multiple birth			
No	381 (21.3)	Reference	–
Yes	9 (18.0)	0.81 (0.34–1.71)	–
HIV-positive subgroup‡			
Antiretroviral treatment			
No	67 (20.4)	Reference	Reference
Yes	15 (18.1)	0.91 (0.45–1.75)	1.14 (0.58–2.26)§
CD4 cell count/mm^3¶			
<200	9 (14.1)	Reference	Reference
200–499	34 (17.2)	1.27 (0.57–2.81)	1.30 (0.58–2.92)§
≥500	33 (28.2)	2.40 (1.07–5.41)	2.55 (1.10–5.90)

*GBS, group B streptococcus; OR, odds ratio; CI, confidence interval; –, not applicable. Adjusted OR derived from logistic regression by use of backward stepwise approach. Final model retained variables with p value <0.1 along with age group and parity because of their known association with GBS carriage.
†χ^2 test for linear trend, p = 0.93.
‡χ^2 test for linear trend, p = 0.01.
§Adjusted model includes age group, first pregnancy vs. second or subsequent pregnancies; antiretroviral treatment, and CD4 count for the HIV-positive subgroup.
¶19 participants had no CD4 count available.

than in the HIV-uninfected women. This association may be consistent with a GBS-specific immune defect, which would concur with what we understand about HIV immunopathology and related capsulate bacteria (*12*). We propose that this higher carriage is obscured at lower CD4 counts by competitive exclusion of GBS in the vagina of women with advanced HIV as a consequence of ecologic changes in the microbial flora (*13*). Increased presence of bacterial vaginosis and anaerobes at low CD4 counts is a feature of HIV, and these conditions may alter the ability of GBS to colonize the vagina (*14*,*15*). Specific studies to investigate anti-GBS immunity and the interactions of the microbial flora in HIV-infected women are required.

Neonatal GBS disease is common in Africa, and disease risk is intimately connected to GBS carriage. The public health consequences of these carriage findings are unclear at present, but further investigation of the interaction of HIV and GBS carriage and risk of neonatal disease is merited, given the recent rise in frequency of GBS infection.

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Incidence Rate for Hantavirus Infections without Pulmonary Syndrome, Panama

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During 2001–2007, to determine incidence of all hantavirus infections, including those without pulmonary syndrome, in western Panama, we conducted 11 communitywide surveys. Among 1,129 persons, antibody prevalence was 16.5%–60.4%. Repeat surveys of 476 found that patients who seroconverted outnumbered patients with hantavirus pulmonary syndrome by 14 to 1.

In the Americas, hantavirus species that occur at low frequency are associated with the severe disease hantavirus pulmonary syndrome (HPS) (*1*,*2*), and species that occur at higher frequency are associated with milder disease (*3*–*5*). In Panama, HPS is caused by the Choclo virus, for which a common rodent, the fulvous pygmy rice rat (*6*), is host. Serum antibody prevalence against this virus is 3%–33% in neighborhoods where HPS cases have occurred (*7*) and 16%–45% according to selected communitywide surveys (*8*). Neutralization-inhibition assays of antibody-positive serum indicated past infections caused by Choclo virus (*9*). To obtain a more accurate incidence of hantavirus infections in Panama, we conducted repeat surveys to identify hantavirus seroconversions during 1- to 3-year intervals between surveys. Our goal was to compare incidence of seroconversion with that of concurrent HPS in the same communities.

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The Study

During 2001–2007, a total of 4 communities (3 in Los Santos Province and 1 in Veraguas Province) within hantavirus-endemic agroecosystems in western Panama were sampled 2–4 times at 1- to 3-year intervals (Table 1). Informed written consent was obtained from all adult participants and from parents or legal guardians of minors. Consent and assent forms were reviewed and approved by institutional ethics review boards at the University of New Mexico, the Gorgas Memorial Institute in Panama City, and the protocol review committee of the International Centers for Infectious Diseases Research program of the National Institute of Allergy and Infectious Diseases. Eligible participants were all adults and children >2 years of age who permanently resided in each community according to the 2000 national census. The reasons for noninclusion in the first and subsequent surveys were absence during the week of the survey and refusal to participate.

A questionnaire administered to the head of household asked for a history of respiratory-related illnesses and hospitalizations within the past 3 years. Venous blood was collected from all family members for serologic testing. Results of the surveys were provided to each participating community through community meetings. Surveillance for HPS was conducted in the same communities as the serosurvey and nationally through reports to the Ministry of Health, and cases of HPS were confirmed by questionnaire. The diagnosis of HPS required finding immunoglobulin (Ig) M in acute-phase serum, detection of Choclo virus RNA in serum by reverse transcription PCR, typical respiratory signs and symptoms, and chest radiographic findings compatible with pulmonary edema.

Heparinized whole blood collected by arm venipuncture was separated by centrifugation; plasma was stored at −20°C until analysis. In binding assays, antibody to all known hantaviruses indigenous to the Americas cross-react with the N protein of Sin Nombre virus (*10*). A strip immunoblot assay for IgG containing recombinant N protein of the 3H226 genotype of Sin Nombre virus was used as described (*10*); the criterion for positivity was a dark band for Sin Nombre N protein at a serum dilution of 1:200. An enzyme immunoassay used recombinant nucleocapsid protein from Sin Nombre virus (*11*); the cutoff value was established at 3 SD above reactivity to a panel of known positive serum. All samples were tested by both assays; the concordance of the enzyme immunoassay and strip immunoblot assay in this study was 97%, and the criterion for seropositivity was a positive reaction in both assays. Loss of antibody in persons with previously positive serum was determined by 2 independent tests with both assays. IgM against hantavirus was not tested. In Panama, all HPS patients tested have had positive reverse transcription PCR results

Table 1. Hantavirus seroprevalence, western Panama*

Community and year of survey	Community population	Persons tested, no. (% of community)	No. (%) IgG positive†	No. undergoing follow-up testing‡	Repeated tests only: no. (%) IgG positive†§
Agua Buena					
2003	175	105 (60)	47 (44.8)	–	–
2004	175	108 (62)	59 (54.6)	75	41 (54.7)
2006	160	102 (64)	61 (60.4)	69	42 (61.8)¶
2007	164	99 (60)	49 (49.5)	55	33 (60.0)#
Isla Cañas					
2001	276	223 (81)	74 (33.2)	–	–
2003	184	120 (65)	56 (46.7)	90	44 (48.9)**
2006	187	120 (64)	63 (52.5)	49	26 (53.1)#
San Jose					
2001	593	486 (82)	80 (16.5)	–	–
2003	454	327 (72)	84 (25.7)	270	70 (25.7)#
Borracherones					
2003	85	61 (72)	19 (31.1)	–	–
2006	93	87 (94)	23 (26.4)	41	11 (26.8)

*Ig, immunoglobulin; –, not applicable.
†No. IgG positive (point prevalence % of seropositivity).
‡No. persons tested in this and previous survey.
§Increase for all 4 communities combined not significant.
¶Increase in seroprevalence significant (p = 0.007) by Fisher exact test.
#Increase for combining Los Santos localities and year, p = 0.0014.
**Increase significant (p = 0.001).

for Choclo genomic RNA in acute-phase blood samples (*9*), and antibody has been detected by both assays.

Data were transferred from field collection forms to a database (Epi Info version 6.04d, Centers for Disease Control and Prevention, Atlanta, GA, USA) for statistical analyses using Epi Info software. Changes in seroprevalence within each community were tested by Fisher exact test for each interval and by longitudinal analysis for all intervals and communities by a generalized estimating equation (*12*). Increases in seroprevalence according to community and year of survey were tested by using analysis of covariance.

The 11 surveys repeatedly sampled 60%–85% of the total population of each community, for a total of 1,838 samples from 1,129 persons. Overall antibody prevalence was 32.9%, varying from 16.5% to 60.4% in individual surveys (Table 1). In each of the 3 Los Santos communities (Agua Buena, Isla Cañas, San Jose), seroprevalence increased annually by ≈5% (Table 1); the overall seroprevalence increases for the combined Los

Table 2. Hantavirus seroconversions and HPS cases, western Panama*

Community and survey intervals	No. undergoing follow-up testing†	No. seroconverted/ no. seronegative‡	No. conversions/ 100 person-years	No. seroreverted/ no. seropositive§	No. HPS cases¶
Agua Buena					
2003–2004	75	8/42		0/33	1
2004–2006	69	6/29		4/40	0
2006–2007	55	5/23		4/32	0
Total		19	15.4	8	1
Isla Cañas					
2001–2003	90	18/64		0/26	0
2003–2006	49	2/24		1/25	0
Total		20	10.0	1	0
San Jose					
2001–2003	270	29/228		1/42	3
Total		29	6.4	1	3
Borracherones					
2003–2006	41	2/26		6/15	1
Total		2	2.6	6	1
Study totals		70	8.2	16	5

*HPS, hantavirus pulmonary syndrome.
†No.persons repeat tested in this and previous survey.
‡ No. seroconverted among subset who were seronegative at the beginning of the interval.
§ No. seroreverted among subset who were seropositive at the beginning of the interval.
¶HPS cases verified by Ministry of Health during interval in community.

Santos communities were significant (Fisher exact test, p = 0.0014). The changes in seroprevalence were community specific (analysis of covariance $F = 5.24$, p = 0.0043), but increases in seroprevalence in the 4 communities combined was not (general estimating equation).

Among the study population, seroconversion was documented for 70 persons, and HPS was diagnosed for 5 other persons in the same communities during the intervals studied (Table 2). In the cohort of 476 persons in all 3 Los Santos communities sampled in 2 back-to-back surveys (Table 2), the 70 seroconversions occurred in persons in all age cohorts and equally among persons of both sexes (data not shown). No person who seroconverted gave a history of HPS-like illness or hospitalization for an acute respiratory illness. A separate study of outpatients with febrile illnesses was conducted in 4 clinics in the hantavirus-endemic area. This study found 48 adults and children with the typical febrile prodrome, unremarkable chest radiographs, and either serum IgM specific for hantavirus nucleoprotein or Choclo virus genomic RNA in the acute-phase blood sample (B. Armien and J.M. Pascale, unpub. data). These findings of symptomatic hantavirus infections confirm previous observations derived from neutralization-inhibition antibody assays (*9*).

The incidence of 70 seroconversions in 857 person-years of observation (Table 2) was equivalent to 8 infections per 100 person-years. The ratio of infection detected by seroconversion to infection resulting in HPS was 14:1. The mean ages of persons who seroconverted (43 years) and those with HPS (43 years) were the same. Undercounting of HPS cases was not likely because HPS is a highly publicized illness throughout Panama, and diagnostic serologic testing is readily available through the Ministry of Health.

A total of 16 seroreversions, compared with 70 seroconversions, occurred among persons in most age cohorts, mean age 48 years. For HPS caused by Sin Nombre and Andes viruses, serum antibody typically persists for years (*13*). Serum antibodies after mild or asymptomatic infections may not persist for many years.

Conclusions

Antibody prevalence surveys are useful for identifying populations and locations at risk, monitoring changes in incidence, and focusing limited public health resources. Determining whether the observed increases in seroprevalence will be sustained requires additional surveys, but this information will be useful as the new agroeconomy increasingly emphasizes the monoculture of products (rice and sugar cane) favorable to rodents (*14*). Nonetheless, the documentation of large numbers of mild or asymptomatic hantavirus infections not progressing to HPS has identified a larger effect of this zoonotic disease.

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Crimean-Congo Hemorrhagic Fever, Afghanistan, 2009

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In response to an outbreak of Crimean-Congo hemorrhagic fever in western Afghanistan, we measured immunoglobulin G seroprevalence among household members and their animals. Seroprevalence was 11.2% and 75.0% in humans (n = 330) and livestock (n = 132), respectively. Persons with frequent exposure to cattle had an elevated risk of being immunoglobulin G positive.

Crimean-Congo hemorrhagic fever (CCHF) is a vector-borne hemorrhagic disease caused by a primarily zoonotic virus infecting a wide range of domestic and wild animals. The main implicated vectors are *Hyalomma* spp. ticks. Transmission of the virus to humans occurs through tick bites; crushing of infected ticks; contact with blood, body fluids, and tissue of patients with CCHF during the acute phase of illness; and contact with blood or tissue of viremic livestock (*1*). In recent years, several CCHF outbreaks were reported in Afghanistan, and the disease persists in neighboring countries (*2*,*3*). In March 1998, an outbreak with 19 cases and 12 deaths (case fatality rate 63.2%) was reported from Takhar Province in the northern part of the country (*4*).

In the fall of 2008, an outbreak occurred in Herat City, western Afghanistan, with ≈60 suspected cases. CCHF was identified in 6 specimens at the Central Public Health Laboratory in Kabul and later confirmed at the laboratories of the US Naval Medical Research Unit No. 3 (NAMRU-3) in Cairo, Egypt. In August 2009, we conducted a cross-sectional seroprevalence survey among livestock-owning households in the same districts of Herat City where the outbreak occurred.

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The Study

The study was conducted in 100 households of 9 affected villages of Engil District on the edge of Herat City. Households were included if they were located on 3 randomly selected transect lines and if members of the households owned either cattle or sheep. Because there are no reliable estimates on seroprevalence of CCHF in Afghanistan, we estimated prevalence of immunoglobulin (Ig) G to be 7% (*2*,*3*). A sample size of 160 persons was required to detect this prevalence at the 95% confidence level. Allowing for cluster sampling at the household level, 320 persons was the target sample size (≈90 households, assuming an average of 3.5 persons ≥15 years of age per household). From each household, all members ≥15 years were surveyed, if they gave consent. Sheep and cattle were selected as livestock types, and 1 or 2 animals per household were randomly selected.

Inclusion criteria for humans were residing in a livestock-owning household, giving informed consent, being ≥15 years of age, willing to answer the risk factor questionnaire, and willing to give 5 mL of blood. For collection of blood samples and vector specimens from sheep and cattle, permission was given by the head of household.

A standardized, structured, and pretested questionnaire that covered individual data for each participant, including personal details, exposure variables, and self-reported disease history, was used. Animal data were collected from each household and included each animal's origin, tick exposure, age, and sex. Information was reported by the head of household or owner of the animal(s).

Blood samples from human participants were collected by trained health workers according to standard procedures. A veterinarian collected blood and tick samples from livestock subjects. Blood samples were centrifuged at room temperature at the local laboratory on the day of collection. Serum was separated, frozen, and transported to Kabul for storage and onward shipment. A sandwich/indirect ELISA detected specific IgG at a 1:100 dilution for all human samples by using the VECTOR-BEST diagnostic kit, (VECTOR-BEST, Novosibirsk, Russia). We have previously compared this kit with an in-house ELISA (US Army Medical Research Institute for Infectious Diseases, Fort Detrick, MD, USA), and the results were comparable in all samples tested (L. Mustafa et al., unpub. data). We used an in-house ELISA using the IbAr 10200 strain of CCHF as antigen (US Army Medical Research Institute for Infectious Diseases) and anti-species IgG horseradish peroxidase–conjugated (KPL Inc., Gaithersburg, MD, USA) to test for IgG in all animal serum specimens. All positive samples were confirmed by duplicate testing in a different run. All ELISA testing was performed at NAMRU-3.

Data were analyzed by using Stata version 8 (StataCorp, College Station, TX, USA). The primary outcome was seropositivity among household members, with secondary outcomes being seropositivity in animals and the presence of virus in ticks. Exposure factors for seroprevalence were identified on an a priori basis and appropriate measures of statistical significance were applied to detect differences at the 95% confidence level. The study was approved by the ethics boards of NAMRU-3 and the Afghan Public Health Institute, Afghanistan.

In total, 330 persons were enrolled from 100 households. Among our sample, IgG seroprevalence was 37/330 (11.2%, 95% confidence interval [CI] 8.0–15.1). Of all the potential explanatory variables, only 2 factors were associated with an elevated risk of IgG positivity: daily contact with cattle (33/264 [12.5%] vs. 1/52 [1.8%]; χ^2 = 5.1, p = 0.02) and exposure to raw animal skins (24/144 [16.7%] vs. 12/176 [6.8%]; χ^2 = 7.7, p = 0.006). Age group was not associated with seroprevalence.

Self reported clinical illness (fever) occurred in 55% of participants over a 5-month reporting period. Among the participants, 20.8% reported that they had had an illness involving bleeding from teeth, gums, and or other parts of the body, but this event was not associated with IgG positivity or with age group.

These results suggest that the risk for CCHF exposure is uniformly high among the population. The oldest age group shows an approximate lifetime risk of exposure and seroconversion of 17% (95% CI 10.2%–25.8%).

Ninety-two cattle and 40 sheep were included, and serologic analysis of their blood samples was conducted. Seroprevalence was 79.1% (95% CI 69.0%–87.1%) among cattle and 75.0% (95% CI 57.0%–88.5%) among sheep. Prevalence was uniformly high regardless of age, sex, or origin of the animals, suggesting that the disease is highly endemic in the livestock population. Among our sample, 84.6% of cattle and 71.5% of sheep had ticks upon inspection by the surveyors. Ticks (n = 259) from domestic animals were predominantly adult *Hyalomma marginatum* (94.6%). Of the total *Hyalomma* ticks collected, 83% were found on cows. Engorged females were found more on cattle than on sheep (43% and 27%, respectively). No association was found between tick infestation and animal serologic results. Most (>85%) animal owners reportedly control ticks by using pesticides. We did not identify virus in tick specimens by PCR (*5*).

Conclusions

Seroprevalence in this population of animal owners is higher than in other reported studies from the region (*3*,*4*), and the risk for exposure appears approximately uniform. This finding indicates that universal control measures are required. The route of transmission to humans is either through the bite of ticks or through contact with infected animals or animal products. This second route of transmission is probably more important in Iran and Afghanistan than tick-borne transmission.

Control of CCHF requires control of the disease vector, and surveillance is necessary to ensure optimum timing of interventions such as livestock dipping or sponging because tick abundance is highly seasonal. Further research on rates of antibody acquisition among humans and animals, virus transmission dynamics, and effectiveness of disease control measures is required. CCHF is a regional public health concern of larger than previously acknowledged significance and requires control mechanisms from both the health and agriculture sectors.

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Extensively Drug-Resistant Tuberculosis in Women, KwaZulu-Natal, South Africa

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To determine whether women in KwaZulu-Natal, South Africa, with drug-resistant tuberculosis (TB) were more likely than men to have extensively drug-resistant TB, we reviewed 4,514 adults admitted during 2003–2008 for drug-resistant TB. Female sex independently predicted extensively drug-resistant TB, even after we controlled for HIV infection. This association needs further study.

Tuberculosis (TB) remains a leading infectious cause of death worldwide (*1*), especially where HIV is endemic (*2*). In industrialized countries, outbreaks of drug-resistant TB (multidrug-resistant [MDR] and extensively drug-resistant [XDR] TB) have occurred predominantly among male patients (*1*). However, in South Africa, where TB and HIV are endemic, aggregate data suggest that a greater proportion of women than men with TB have MDR TB (*3*).

In South Africa, HIV infection is more prevalent among women than men. Nationally, women 25–29 years of age have the highest (32.7%) HIV prevalence; for men, prevalence peaks at age 30–34 years (25.8%) (*4*). KwaZulu-Natal Province, the epicenter of the HIV/AIDS epidemic in South Africa, has a high incidence of hospital admissions for MDR TB and XDR TB and >70% HIV co-infection among MDR TB patients (*5*). Since 2003, fueled by a generalized HIV epidemic, TB incidence in South Africa (*1*) and hospital admissions for MDR TB in KwaZulu-Natal have doubled (*5*). Observational studies of MDR TB and XDR TB in South Africa report higher proportions of female patients with MDR TB or XDR TB (*6–9*). We conducted this study to determine whether women in KwaZulu-Natal with drug-resistant TB were more likely than men to have XDR TB, even after we controlled for HIV and other factors.

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The Study

The study design has been described (*5*). Briefly, we retrospectively reviewed adult MDR TB and XDR TB patients admitted during 2003–2008 for treatment initiation to King George V Hospital (KGVH), a public TB-referral hospital in KwaZulu-Natal. Admitting physicians and staff collected data routinely. During the study period, KGVH was the only public hospital in the province authorized to initiate treatment for MDR TB and XDR TB, and all therapy was initiated on an inpatient basis.

All patients ≥18 years of age who had culture-confirmed MDR TB or XDR TB with standard drug susceptibility testing were included. Repeat admissions were excluded. MDR TB and XDR TB were defined according to standard definitions (*10*). Ethics review committees at the University of KwaZulu-Natal and Boston University (Boston, MA, USA) approved the study protocol.

We used Fisher exact or χ^2 tests to compare categorical variables. Medians were compared by using the Wilcoxon-Mann-Whitney U test. Univariate and multivariate logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs). Statistically significant variables or variables that caused >10% change in the univariate OR were included in the multivariate model. Interaction terms were assessed by using Wald tests, with $p<0.2$ considered significant. Test for trend was performed by using the Cochran-Armitage test. Analysis was performed with SAS version 9.3 software (SAS Institute, Inc., Cary, NC, USA).

A total of 4,941 patients with MDR TB or XDR TB from throughout KwaZulu-Natal were admitted for initiation of drug-resistant TB treatment to KGVH during the study period (Figure). Among 4,514 eligible patients with MDR TB or XDR TB, women were younger (median age [interquartile range] 32 [26–39] vs. 36 [30–44] years), more likely to be HIV infected (65% vs. 47%), and more likely than men to be receiving antiretroviral therapy (ART) (51% vs. 43%) (Table 1). Women with drug-resistant TB were more likely than men with drug-resistant TB to have XDR TB ($p<0.0001$).

On univariate analysis, HIV status, ART (among HIV-infected), female gender, previous TB treatment, and year of admission to KGVH were significantly associated with XDR TB (Table 2). On multivariate analysis, only female gender (OR 1.38, 95% CI 1.11–1.73), previous TB treatment (OR 2.16, 95% CI 1.09–4.28), and year of admission were independently associated with XDR TB. In the HIV strata, ART was not significantly associated

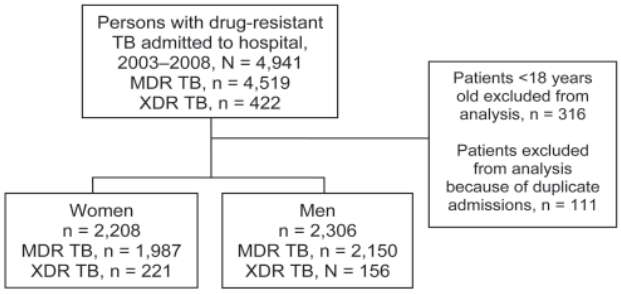

Figure. Flow diagram for patients with multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) admitted to King George V Hospital, KwaZulu-Natal, South Africa, 2003–2008.

with XDR TB after adjustment was made for confounding variables. Confounding of ART and XDR TB by year of admission among HIV co-infected persons most likely resulted from increased XDR TB case finding after a highly publicized XDR TB outbreak in 2006 and increased ART use after public health rollout of ART in KwaZulu-Natal in 2004. The interaction terms HIV × gender, gender × age (categorical), and health care worker (HCW) × gender were not significantly associated with XDR TB.

Most (59%) patients admitted with XDR TB were women, which did not change significantly during the study period (test for trend p = 0.68). For MDR TB, the data showed increasingly more female MDR TB patients admitted over the study period (p<0.001) (online Appendix Table, www.cdc.gov/EID/content/17/10/11-0105-appT.htm).

Conclusions

Our major finding was that women admitted with drug-resistant TB to KGVH were 38% more likely than men to have XDR TB. This association remained significant after adjustment for potential confounding variables, including HIV status. Temporal analysis showed persistently more women with XDR TB and increasing proportions of women with MDR TB during the study period. Together these data support the notion that the epidemic of drug-resistant TB predominantly affects women in KwaZulu-Natal.

Supporting context comes from observational studies of drug-resistant TB in South Africa. In South African studies, higher percentages of XDR TB (50%–56%) than MDR TB patients (43%–53%) are women (*6*–*9*). However, studies from low-prevalence HIV settings report fewer women with drug-resistant TB. In a study in the United States, few patients with MDR TB (36%) or XDR TB (38%) were female (*11*). Similarly, in cohorts from Latvia, Peru, and Russia, lower percentages of patients with MDR TB (17%–40%) and XDR TB (29%–35%) were female (*12*–*14*). Gender differences in drug-resistant TB in areas of HIV endemicity and low prevalence suggest a possible effect of the AIDS epidemic on prevalence of drug-resistant TB in women.

Table 1. Characteristics of 4,514 adults ≥18 years of age with MDR TB and XDR TB admitted to King George V Hospital, KwaZulu-Natal, South Africa, 2003–2008*

Characteristic†	No. (%) women, n = 2,208	No. (%) men, n = 2,306	p value
HIV status			
Positive	1,431 (64.8)	1,083 (47.0)	<0.0001
Negative	394 (17.8)	642 (27.8)	
Unknown	383 (17.3)	581 (25.2)	
HIV positive and ART			
Yes	731 (51.1)	465 (42.9)	<0.0001
No	700 (48.9)	618 (57.1)	
Health care worker			
Yes	180 (8.2)	51 (2.2)	<0.0001
No	2,028 (91.8)	2,255 (97.8)	
Previous treatment			
Yes	1,987 (95.4)	2,127 (94.4)	0.1305
No	96 (4.6)	127 (5.6)	
Year of hospital admission			
2003	188 (8.5)	278 (12.1)	0.0011‡
2004	195 (8.8)	234 (10.1)	
2005	272 (12.3)	283 (12.3)	
2006	358 (16.2)	350 (15.2)	
2007	590 (26.7)	593 (25.7)	
2008	605 (27.4)	568 (24.6)	
Type of TB			
MDR	1,987 (90.0)	2,150 (93.2)	<0.0001
XDR	221 (10.0)	156 (6.8)	

*MDR, multidrug-resistant; TB, tuberculosis; XDR, extensively drug-resistant; ART, antiretroviral therapy.
†Median patient age (interquartile range): women, 32 y (26–39 y); men, 36 y (30–44 y); p<0.0001.
‡Cochran-Armitage test for trend.

Table 2. Risk factors for XDR TB and MDR TB among adults ≥18 years of age admitted to King George V Hospital with drug-resistant TB, KwaZulu-Natal, South Africa, 2003–2008*

Risk factor	No. (%) with XDR TB, n = 377	No. (%) with MDR TB, n = 4,137	OR (95% CI)	
			Univariate analysis	Multivariate analysis
Sex				
F	221 (58.6)	1,987 (48.0)	1.53 (1.24–1.90)	1.38 (1.11–1.73)
M	156 (41.4)	2,150 (52.0)	Reference	Reference
Age, y†				
18–35	204 (54.1)	2,265 (54.7)	0.98 (0.79–1.20)	–
≥36	173 (45.9)	1,872 (45.3)	Reference	
HIV status‡				
Positive	259 (68.7)	2,255 (54.7)	1.43 (1.10–1.87)	1.19 (0.90–1.56)
Negative	77 (20.4)	959 (23.2)	Reference	Reference
Unknown	41 (10.9)	923 (22.3)	0.55 (0.38–0.82)	0.69 (0.46–1.04)
Previous TB treatment				
Yes	359 (97.6)	3,755 (94.6)	2.27 (1.16–4.47)	2.16 (1.09–4.28)
No	9 (2.5)	215 (5.4)	Reference	Reference
HIV positive and ART‡				
Yes	149 (57.5)	1,047 (46.4)	1.56 (1.21–2.03)	–
No	110 (42.5)	1,208 (53.6)	Reference	
Year of admission				
2003	6 (1.6)	460 (11.1)	Reference	Reference
2004	5 (1.3)	424 (10.2)	0.90 (0.27–2.98)	0.96 (0.27–3.33)
2005	36 (9.5)	519 (12.5)	5.32 (2.22–12.74)	5.03 (1.94–12.99)
2006	78 (20.7)	630 (15.2)	9.49 (4.10–21.97)	9.26 (3.71–23.13)
2007	137 (36.3)	1,046 (25.3)	10.04 (4.40–22.91)	9.14 (3.70–22.60)
2008	115 (30.5)	1,058 (25.6)	8.33 (3.64–19.07)	7.45 (3.00–18.50)

*XDR, extensively drug-resistant; TB, tuberculosis; MDR, multidrug-resistant; OR, odds ratio; CI, confidence interval; ART, antiretroviral therapy.
†Median age (interquartile range): MDR TB patients 34 y (28–42 y); XDR TB patients, 35 y (29–42 y).
‡Univariate analysis restricted to HIV-positive persons only. ART use excluded from multivariate analysis because persons receiving ART must be HIV infected.

Our study has several limitations. We lacked details about hospital admission and factors associated with referral. Women with XDR TB might have been preferentially referred compared with men with XDR TB, women with MDR TB, or both. We lacked data on HIV factors, such as CD4 T-cell counts, ART adherence, and viral load. Similarly, we lacked data on previous TB treatment, medications, adherence, and outcome. Finally, as a hospital-based retrospective study, factors associated with survival to hospital admission, decisions to seek care, and referral patterns may introduce bias.

Potential causes of the association between female gender and XDR TB are not known. HIV-related factors could explain the association of XDR TB and female gender. For example, women with drug-resistant TB may be more adherent to ART, leading to improved survival, and therefore increased time for XDR TB to develop. Factors associated with secondary development of XDR TB, such as TB medication adherence or previous MDR TB treatment, could explain the association between XDR TB and female gender (*15*). Factors associated with exposure to drug-resistant TB strains, including location and duration of exposure, could explain the association of XDR TB and female gender. For example, women are more likely to participate in formal and informal care work, with potential exposure to drug-resistant TB strains, and therefore primary XDR TB might be more likely to develop (*5*).

These results may have major policy implications for TB control in KwaZulu-Natal and South Africa. Because women are more likely to have XDR TB in KwaZulu-Natal, efforts should be made to develop gender-sensitive interventions to improve diagnosis, treatment, and prevention for drug-resistant TB and HIV. Decentralization of drug-resistant TB treatment may better accommodate women in conjunction with their work, family, and child-rearing responsibilities. Further studies are needed to confirm the magnitude and determinants of the association between female gender and XDR TB.

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Clostridium difficile Infection in Outpatients, Maryland and Connecticut, USA, 2002–2007

Jon Mark Hirshon, Angela D. Thompson, Brandi Limbago, L. Clifford McDonald, Michelle Bonkosky, Robert Heimer, James Meek, Volker Mai, and Christopher Braden

Clostridium difficile, the most commonly recognized diarrheagenic pathogen among hospitalized persons, can cause outpatient diarrhea. Of 1,091 outpatients with diarrhea, we found 43 (3.9%) who were positive for *C. difficile* toxin. Only 7 had no recognized risk factors, and 3 had neither risk factors nor co-infection with another enteric pathogen.

In the United States, ≈375 million episodes of acute diarrhea occur annually (*1*). Among hospitalized persons, toxin-producing *Clostridium difficile* is a primary diarrheagenic pathogen, usually as a consequence of normal bowel flora distortion caused by antimicrobial drug therapy (*2*,*3*). *C. difficile* infection (CDI) complicates and prolongs hospital stays, leading to increases in health care costs, illness, and death. Recent reports suggest increases in community-onset CDI among persons without recent antimicrobial drug treatment or hospitalization. We describe a prospective evaluation of CDI in persons with diarrhea who visited emergency departments (EDs) and ambulatory primary care clinics in Baltimore, Maryland, and New Haven, Connecticut, and identify microbiologic causes and epidemiologic characteristics of diarrhea. This report highlights cases of outpatient CDI, identifies factors associated with infection, and describes molecular strain characterization.

Patients seeking medical attention for community-onset diarrheal illnesses were enrolled from May 2002 through September 2004 in the EDs and ambulatory clinics at Yale–New Haven Hospital (New Haven, CT, USA) and from May 2002 through July 2007 at EDs and clinics affiliated with the University of Maryland (Baltimore, MD, USA) (*4*).

Informed consent for stool sample collection, initial and follow-up patient interviews, and medical records review was obtained from primarily urban and suburban residents, or parents/guardians for minors, who sought treatment for self-identified primary or secondary diarrhea. This research was approved by the institutional review boards at all participating institutions.

Participants were interviewed at outpatient clinics to assess health status, symptoms, and potential exposures to enteric pathogens, and at follow-up to determine the duration of diarrhea, whether treatment was administered, or whether hospitalization resulted from the initial visit. Stool samples collected during the visit or provided within 48 h and kept cool were homogenized and transferred into multiple vials for storage at –80°C.

An outpatient CDI case was defined in an outpatient with diarrhea whose stool was positive for *C. difficile* toxins by enzyme immunoassay (TOX A/B II ELISA; TechLab, Blacksburg, VA, USA). Presumptive non–health care–associated (NHA) CDI was defined by the absence of an overnight stay at an inpatient healthcare facility over the previous month.

Traditional risk factors for CDI that were investigated included antimicrobial drug use within the past month, age ≥65 years, serious underlying illness/weakened immune system, history of bowel or ulcer surgery, colon disease, previous CDI, and recent hospitalization. Statistical analysis was done by using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA). All p values reported are 2-sided, with no correction for multiple comparisons; $p<0.05$ was considered significant.

C. difficile toxin–positive stool specimens, in 1-mL aliquots, were shipped frozen to the Centers for Disease Control and Prevention (Atlanta, GA, USA) anaerobe laboratory for culturing by direct inoculation onto cycloserine cefoxitin fructose agar (CCFA) or ethanol shock, followed by CCFA inoculation. Cultures were incubated for 48–72 h at 35°C under anaerobic conditions and examined for characteristic yellow-green fluorescence under long-wave ultraviolet light and CCFA*p*-cresol odor. *C. difficile* colonies were confirmed with indole (negative) and PRO disk (positive; Remel, Lenexa, KS, USA) tests.

Pulsed-field gel electrophoresis was performed on *C. difficile* genomic DNA digested with *Sma*I, and toxinotyping was performed (*5*). Binary toxin was assayed by PCR for *cdtB* (*6*). Deletions in *tcdC* were detected (*7*).

C. difficile toxin tests were performed on 1,091/1,197 stool specimens; 43 (3.9%) of these case-patients met the case definition for outpatient CDI. The mean age of these

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Table 1. Patient risk factors for CDI compared with those of other patients with diarrhea without CDI, Maryland and Connecticut, USA, 2002–2007*

Risk factor	No. (%) patients with CDI, n = 43	No. (%) patients with diarrhea but not CDI, n = 1,048	p value†
Illnesses potentially affecting immune status			
Lupus	1 (2.3)	4 (0.4)	0.06
Cancer under active treatment	3 (7.0)	40 (3.8)	0.30
HIV/AIDS	2 (4.7)	43 (4.1)	0.86
History of organ transplant	2 (4.7)	22 (2.1)	0.26
Chronic obstructive pulmonary disease (on prednisone)	1 (2.3)	NA	NA
Illnesses potentially affecting gastrointestinal function			
Crohn disease	0 (0)	14 (1.3)	0.45
Ulcerative colitis	1 (2.3)	19 (1.8)	0.81
Prior bowel or ulcer surgery	6 (14.0)	69 (6.6)	0.06
Any medical or surgical condition	15 (34.9)	176 (16.8)	0.002
Hospitalized within prior month	14 (32.6)	92 (8.8)	<0.001
Antimicrobial drug therapy within prior month	27 (62.8)	231 (22.0)	<0.001
No hospitalization or antimicrobial drug therapy within prior month and no predisposing condition	7 (16.3)	698 (66.6)	<0.001

*CDI, *Clostridium difficile* infection; NA, not available.
†Uncorrected χ^2.

case-patients was 43.7 years (range 4 months–88 years). Outpatient CDI case-patients were younger at Yale because a significantly greater proportion of toxin-positive children were recruited at Yale (45.5%) than at the University of Maryland (15.6%) (p = 0.04). The 43 outpatient CDI case-patients included 5 infants <1 year of age, 5 children 1–18 years of age, 23 adults 19–64 years of age, and 10 adults ≥65 years of age; 21 were Caucasian, 18 were African American, and 4 were of other or unknown race/ethnicity (22 male and 21 female case-patients).

Most case-patients (36/43, 83.7%) had a recognized underlying risk factor. Twenty-seven (62.8%) had received systemic antimicrobial drugs, including ciprofloxacin, gaitifloxacin, amoxicillin, ampicillin/sulbactam, piperacillin/tazobactam, cefpodoxime, vancomycin, clindamycin, metronidazole, erythromycin, or trimethoprim/sulfamethoxazole within the preceding month; 14 (32.6%) had been hospitalized; and 15 (34.9%) had chronic illnesses or had undergone bowel surgery that potentially affect immune status or gastrointestinal function (Table 1). Two persons, 1 with AIDS and 1 who underwent a previous bowel resection for diverticulitis, had been treated in the past month for CDI. Only 7 (16.3%) patients had NHA-CDI infections without identified risk factors; 3 were infants (<1 year), 1 was a child (1–18 years), 3 were adults (19–64 years), and none were elderly (≥65 years) (Table 2).

The 43 outpatient CDI case-patients were compared with the other 1,048 persons in which *C. difficile* toxin had not been detected. Persons with CDI were, on average, significantly older than others with diarrhea, 43.7 years vs. 29.2 years, respectively (p<0.01). Outpatient CDI case-patients were more likely than *C. difficile*–negative patients to have medical or surgical conditions (34.9% vs.16.8%, p<0.001), been recently hospitalized (32.6% vs. 8.8%, p<0.001), or to have used antimicrobial drugs (62.8% vs. 22.0%, p<0.001).

Co-infections with other enteric pathogens were common among CDI case-patients, including *C. perfringens* (3), rotavirus (5), norovirus (3), sapovirus (2), and 1 each with hookworm, *Bacillus cereus*, astrovirus, and adenovirus. The likelihood of co-infection was similar in patients with (12/36 [33.3%]) and without (3/7 [42.9%]) risk factors (p>0.1).

Of 43 *C. difficile* toxin–positive stools initially tested, 39 stool samples were submitted to the Centers for Disease

Table 2. Characteristics of CDI case-patients who had no identified risk factors, Maryland and Connecticut, USA, 2002–2007*

Patient ID	Recruitment site	Age/sex	Race/Ethnicity	Other medical conditions	Co-infections
1	Maryland	62 y/M	White	Hypertension, GERD, COPD/asthma, depression/anxiety	
2	Maryland	6 mo/M	White	Reflux	*C. perfringens*, rotavirus
3	Yale	20 mo/M	Hispanic	None	Norovirus
4	Yale	5 mo/M	White	None	
5	Yale	28 y/F	Black	None	Rotavirus
6	Yale	34 y/F	Hispanic	Polycystic ovary disease, diabetes, GERD	
7	Yale	4 mo/M	Hispanic	None	Norovirus

*CDI, *Clostridium difficile* infection; GERD, gastroesophageal reflux disease; COPD, chronic obstructive pulmonary disease.

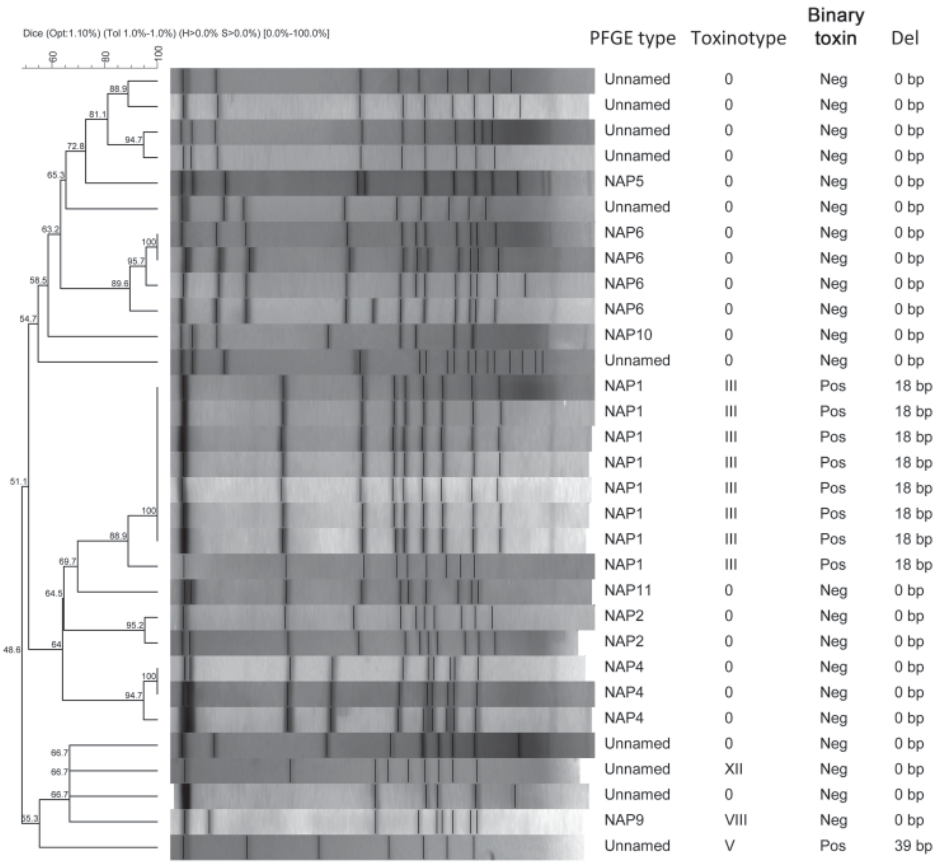

Figure. Characteristics of isolates obtained from patients with *Clostridium difficile* infection, Maryland and Connecticut, USA, May 2002–July 2007. PFGE, pulsed-field gel electrophoresis.

Control and Prevention for anaerobic culture and *C. difficile* was isolated from 31 samples. Binary toxin was identified in 12 (38.7%). Pulsed-field gel electrophoresis identified 15 different types (Figure). No associations were found between risk factors, including age, and strain or toxinotype (data not shown).

NHA-CDI has been recognized for >12 years, and recent reports suggest that disease occurs without patient's known exposure to antimicrobial drugs or other previously identified risk factors (*2*,*8*–*13*). Although we found a proportion of *C. difficile*–positive diarrheal stools similar to that of 2 other recent prospective studies that used confirmatory culture (i.e., 1.5%–3.9%) for outpatient CDI (*7*,*13*,*14*), we also found a lower proportion of outpatient CDI cases without recognized risk factors of recent hospitalization, chronic medical conditions, recent antimicrobial drug exposure, or co-infection than did those studies.

One limitation of our study was using retrospective self-reporting for assessment of hospitalizations or antimicrobial drug use in the previous month, which potentially can result in recall bias. Also, antimicrobial drug therapy was assessed for only 4 weeks before diarrhea onset; exposure to antimicrobial drugs for a period longer than 1 month before patient seeks treatment may present a risk for CDI. In addition, this study was conducted at 2 urban centers in the eastern United States and may not be generalizable to other locations or clinical settings. Finally, although enzyme immunoassay detection for *C. difficile* was the standard of care at the time of the study, it is now considered too insensitive to be used as a stand-alone diagnostic test (*15*).

In summary, we detected toxigenic *C. difficile* in a similar proportion of patients to those reported in other studies of CDI. However, all but 3 patients had either known risk factors for CDI or other pathogens potentially responsible for their illness; 1 was <1 year of age. *C. difficile* isolates responsible for outpatient CDI are genetically diverse. An evolving picture of widespread, frequent CDI among outpatients without risk factors should be tempered by these findings.

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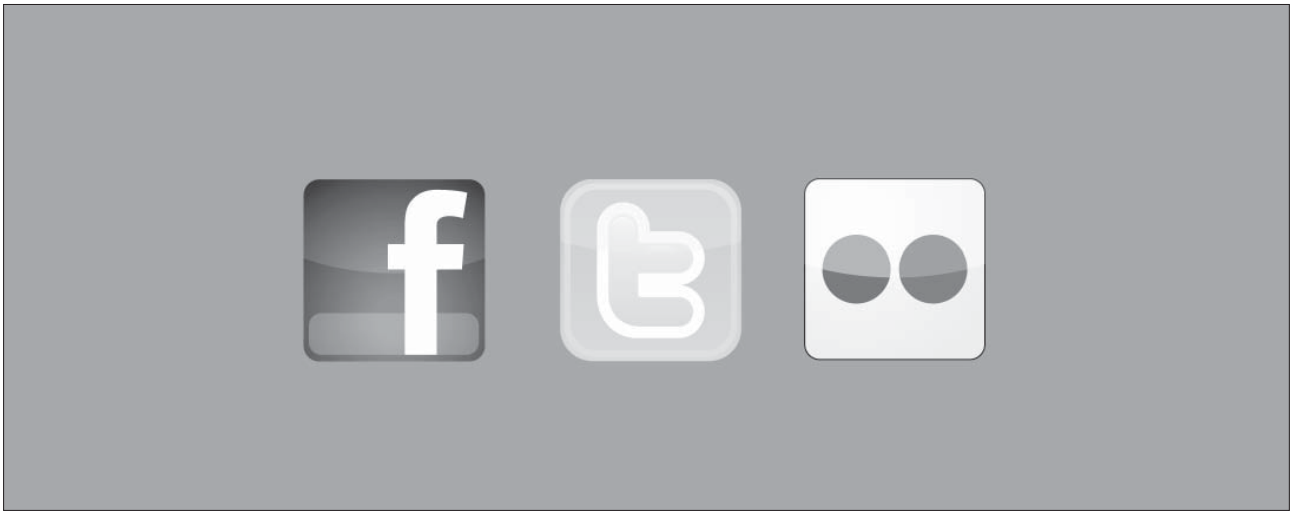

CTX-M-15–producing Enteroaggregative *Escherichia coli* as Cause of Travelers' Diarrhea

Elisabet Guiral, Eva Mendez-Arancibia, Sara M. Soto, Pilar Salvador, Anna Fàbrega, Joaquim Gascón, and Jordi Vila

Travelers' diarrhea is a major public health problem. From patients in whom diarrhea developed after travel to India, 5 enteroaggregative *Escherichia coli* strains carrying β-lactamase CTX-M-15 were identified; 3 belonged to clonal complex sequence type 38. This β-lactamase contributes to the multidrug resistance of enteroaggregative *E. coli*, thereby limiting therapeutic alternatives.

Travelers' diarrhea remains a major public health problem, causing substantial illness and disability. Almost 50% of patients with travelers' diarrhea require treatment with antimicrobial drugs because of persistence or severity of signs and symptoms (*1*). Enteroaggregative *E. coli* (EAEC) is among the most common diarrheagenic *E. coli* pathotypes recognized (*2*). The first-choice agents for treating EAEC infections are quinolones, rifaximin, azithromycin, and cephalosporins. However, the number of pathogenic *E. coli* strains resistant to multiple antimicrobial agents has increased, and resistance to third-generation cephalosporins (e.g., ceftazidime, ceftriaxone, or cefotaxime) associated with production of extended-spectrum β-lactamases (ESBLs) limits therapeutic options (*3*).

Although ESBL production has mainly been shown in extraintestinal *E. coli* infections, studies concerning effects of ESBLs in intestinal *E. coli* infections are scarce. The worldwide spread of CTX-M-15 type ESBLs has led these β-lactamases to replace TEM- and SHV-type ESBLs in Europe, Canada, and Asia and become one of the major groups of ESBLs studied. Of the different CTX-M–type ESBLs, CTX-M-15 has become the most widely distributed enzyme worldwide. It was first identified in an isolate from India in 1999 and thereafter became prevalent around the world (*4*). CTX-M-15 enhances hydrolytic activity against ceftazidime (*5*). A particular clone of CTX-M-15–producing *E. coli*, characterized by phylogenetic type (phylotype) B2 and sequence type 131 (ST131), seems to be largely responsible for international epidemics of CTX-M–producing *E. coli* (*6*). Sequence types (STs) are grouped into clonal complexes by their similarity to a central allelic profile.

ST131 is a singleton and therefore does not belong to a clonal complex (*7*). Molecular epidemiologic studies have suggested that the sudden increase in CTX-M-15–producing *E. coli* worldwide was mainly caused by this single clone (ST131) and that foreign travel to high-risk areas, such as the Indian subcontinent, might play a partial role in the spread of this clone across continents (*8*). The $bla_{\text{CTX-M-15}}$ gene is usually found downstream from the insertion sequence IS*Ecp1*, which may be involved in the clone's dissemination and expression (*9*). We describe molecular epidemiology and plasmid analyses of 5 CTX-M-15–producing EAEC isolates from patients with travelers' diarrhea who had traveled from Spain to India.

The Study

The study included all patients with diarrhea who visited the Tropical Medicine Unit of Hospital Clinic in Barcelona, Spain, during 2005 and 2006. Patients with diarrhea that started during or shortly after (<5 days) a stay in a developing country were eligible. After the participants provided informed consent, clinical and epidemiologic data were collected.

Among all eligible participants, infection with EAEC and no other enteropathogen was found for 51. Of these 51 EAEC isolates, 5 from patients who had traveled to India were resistant to third-generation cephalosporins. Resistance phenotypes indicated ESBL production. MICs for antimicrobial agents and susceptibility class were determined by using the Clinical and Laboratory Standards Institute breakpoints guideline (Table 1). All strains were resistant to penicillins; second-, third-, and fourth-generation cephalosporins; and all β-lactamase–inhibitor combinations except piperacillin/tazobactam. Apart from β-lactam susceptibility, the strains showed resistance to other classes of antimicrobial agents, such as fluoroquinolones, tetracyclines, and monobactams (aztreonam). Positive amplification with specific primers and sequencing for the $bla_{\text{CTX-M-15}}$ gene provided positive genotypic confirmatory test results for ESBL production.

The epidemiologic relationships among the 5 strains were studied by repetitive sequence–based PCR, pulsed-field gel electrophoresis, and multilocus sequence typing (*10*,*11*). The PCR and pulsed-field gel electrophoresis genomic fingerprinting showed that the 5 strains were not epidemiologically related (Figure 1). However, multilocus

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Table 1. Susceptibility of 5 enteroaggregative *Escherichia.coli* strains that produced diarrhea in patients returning from India, 2005–2006*

Strain	Antimicrobial agent																							
	AM	PR	AG	P/T	A/S	FU	FOX	FZ	PIM	CTX	CAZ	GN	AK	TB	F	IMI	ME	AZ	CIP	NOR	LEV	TE	SXT	CL
HC19	R	R	R	S	R	R	I	R	R	R	R	R	S	R	S	S	S	R	R	R	R	S	R	S
HC64	R	R	R	S	R	R	I	R	R	R	R	S	S	R	S	S	S	R	R	R	R	R	R	S
HC67	R	R	R	S	R	R	I	R	R	R	I	R	R	R	S	S	S	R	R	R	R	R	R	S
HC74	R	R	R	S	R	R	I	R	R	R	R	S	R	R	S	S	S	R	R	R	R	R	R	R
HC76	R	R	I	S	R	R	S	R	I	R	S	S	S	S	S	S	S	I	R	R	R	R	R	S

*AM, ampicillin; PR, piperacillin; AG, amoxiclavulanic acid/augmentin; P/T, piperacillin/tazobactam; A/S, ampicillin/sulbactam; FU, cefuroxime; FOX, cefoxitin; FZ, cefazoline; PIM, cefepime; CTX, cefotaxime; CAZ, ceftazidime; GN, gentamicin; AK, amikacin; TB, tobramycin; F, fosfomycin; IMI, imipenem; ME, meropenem; AZ, aztreonam; CIP, ciprofloxacin; NOR, norfloxacin; LEV, levofloxacin; TE, tetracycline; SXT, cotrimoxazole; CL, chloramphenicol; R, resistant; S, sensitive; I, intermediate.

sequence typing identified 2 clonal complexes: ST38 (3 strains) and ST10 (1 strain). The fifth strain could not be classified into any clonal complex (Table 2).

E. coli strains were classified into phylogenetic groups by multiplex PCR, described by Clermont et al. (*12*). The 3 strains in clonal complex ST38 belonged to the potentially virulent phylogenetic group D; the other 2 belonged to group B2 (Table 2).

A PCR method was used to detect genes encoding for typical EAEC virulence factors (*2*). These genes include *aggA* and *aafA* (encoding for adhesions); *aap* (for dispersin); *aatA* (for TolC); *aggR* (for regulation of aggregation); *astA*, *set1A*, and *sen* (for toxins), *fyuA* (for iron recruitment); *agn43* (for antigen 43); and genes encoding for serine protease autotransporter toxins such as *pet* and *sat*. Gene *aatA* was detected in the 5 strains, whereas *aap*, *aggR*, and *aggA* had positive amplification for only 2 of the strains belonging to ST38. The other genes detected are shown in Table 2. EAEC was also identified by typical adherence to HEp-2 cells.

To determine the genetic environment of the $bla_{\text{CTX-M-15}}$ gene, we designed an inverse PCR. We designed the primers by studying the gene sequence and were directed outside the gene. The IS*Ecp1* insertion sequence was upstream from the $bla_{\text{CTX-M-15}}$ gene, which was also confirmed by PCR of the specific insertion sequence. To confirm the possible relationship between IS*Ecp1* and the resistance $bla_{\text{CTX-M-15}}$ gene, we conducted a PCR with the forward primer for the IS*Ecp1* and the reverse primer for the $bla_{\text{CTX-M-15}}$ gene.

For plasmid extraction of the 5 isolates, we used the method of Kado and Liu (*13*). Only 3 strains had plasmids ranging from 93 kb to 170 kb (Figure 2, panel A). To confirm the absence of plasmids in the 2 strains, we conducted S1 digestion of the strains, resolving chromosomal DNA from plasmidic DNA. Southern blot of this digestion showed that the $bla_{\text{CTX-M-15}}$ gene was chromosomally located in these 2 strains, as was the *aatA* gene (usually found in the plasmid contained in EAEC strains) (data not shown). Finally, the location of the $bla_{\text{CTX-M-15}}$ gene in the 3 plasmid-containing strains was analyzed by using Southern blot from the plasmid extraction. The $bla_{\text{CTX-M-15}}$ gene was located in a plasmid in the 3 strains. The size of the plasmid containing CTX-M-15 varied in each strain (Figure 2, panel B). Plasmids with specific known molecular weight were used to provide a range of the size of the plasmids studied.

Conclusions

We identified several features concerning the molecular epidemiology of CTX-M-15–producing EAEC isolates in India. First, all strains belonged to phylogenetic groups D and B2, the 2 groups most commonly found with *E. coli* infections (*14*). Second, not finding ST131 suggests that ST131 might not be the most common ST among EAEC strains from India and that clonal complex ST38 might play a large role in causing infectious intestinal diseases. Third, the $bla_{\text{CTX-M-15}}$ gene is not only located in the plasmid but may also be in the chromosome. However, previous reports have shown that $bla_{\text{CTX-M-15}}$ is consistently linked with IS*Ecp1*, which means that the chromosomal location might have originated from a previous plasmid location that was part of either a transposon or a cassette within an integron (*9*). It is also worth noting that the size of the plasmids containing the $bla_{\text{CTX-M-15}}$ gene was not the same in all strains, indicating that this gene may be located in different types of plasmids.

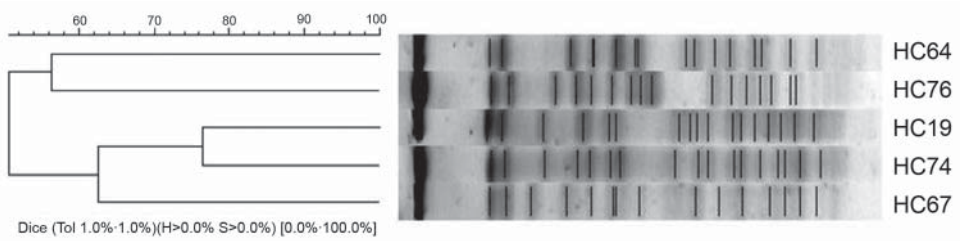

Figure 1. Cluster analysis of the enteroaggregative *Escherichia coli* strains from the pulsed-field gel electrophoresis fingerprinting.

Table 2. Analysis results for 5 enteroaggregative *Escherichia coli* strains that produced travelers' diarrhea in patients returning from India, 2005–2006*

Strain	PFGE type	MLST clonal complex	Phylotype	Genes encoding for virulence factors	$bla_{CTX-M-15}$ location
HC19	A	ST38	D	*aat, aap, aggR, aggA*	Chromosome
HC64	B	None	B2	*aat, astA,sat*	Plasmid
HC67	C	ST38	D	*aat, astA*	Plasmid
HC74	A_1	ST38	D	*aat, aap, aggR, aggA, afn43, fyuA*	Chromosome
HC76	D	ST10	B2	*aat, fyuA*	Plasmid

*PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence type; ST, sequence type.

This evidence of widespread distribution and flexibility of the $bla_{CTX-M-15}$ gene highlights the need to develop appropriate means to control dissemination of this gene and associated resistance genes. Epidemiologic surveillance and correct use of antimicrobial agents will help prevent the steady increase of antimicrobial drug resistance worldwide.

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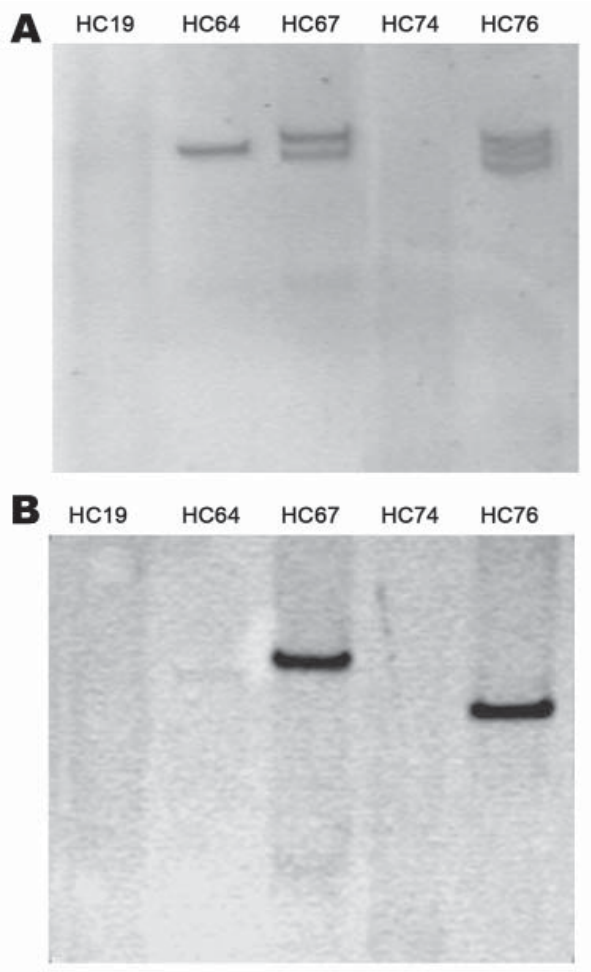

Figure 2. Plasmidic profile of the enteroaggregative *Escherichia coli* strains (A) and Southern blotting of the $bla_{CTX-M-15}$ gene (B).

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Placental Transmission of Human Parvovirus 4 in Newborns with Hydrops, Taiwan

Mao-Yuan Chen, Shiu-Ju Yang, and Chien-Ching Hung

In studying the epidemiology of parvovirus 4 (PARV4) in Taiwan, we detected DNA in plasma of 3 mothers and their newborns with hydrops. In 1 additional case, only the mother had PARV4 DNA. Our findings demonstrate that PARV4 can be transmitted through the placenta.

Transmission routes of human parvovirus 4 (PARV4), a recently discovered member of the *Parvoviridae* family (*1*), are not fully understood; studies have suggested that PARV4 is transmitted predominantly through the parenteral route (*2*,*3*). To study the epidemiology of PARV4 infection in Taiwan, we developed an in-house PARV4 immunoblot (*4*). During the process, we found regions of higher similarity in amino acid sequence between PARV4 and parvovirus B19 virus (B19V). They are LPG***T***NYVGPGN***E***L (B19V VP1, aa 125–137) LPG***Y***NYVGPGN***P***L (PARV4 open reading frame [ORF] 2, aa 219–231) and Y***K***YPYVLG***QG***QDTL (B19V VP2, aa 157–170) Y***D***YPYVLG***HN***QDTL (PARV4 ORF2, aa 499–512).

To exclude the possibility of antibody cross-reaction between PARV4 and B19V, we tested plasma samples sent to our laboratory for confirmation of B19V infection with PARV4 immunoblot. Unexpectedly, we detected PARV4 DNA in plasma from a mother and her newborn with hydrops. Therefore, we examined samples from 5 additional infants with hydrops.

The Study

During 2000–2009, our laboratory received blood samples from 6 infants with nonimmune idiopathic hydrops (Table 1). Paired mother–newborn plasma samples from 4 infants were available for this study; plasma from either the mother or newborn was missing in 2 instances. None of the blood samples from the newborns was cord blood. All infants (case-patients) had at least 2 of the following conditions: ascites, pleural effusion, pericardial effusion, skin edema, or polyhydramnios.

Antibodies to PARV4 and B19V were tested by immunoblots. DNA of PARV4 and B19V was detected by seminested and nested PCR, respectively. PARV4 immunoblot and PCR were performed according to the methods in our previous report (*4*). The B19V immunoblot and PCR are described in the online Technical Appendix (www.cdc.gov/EID/content/17/10/10-1841-Techapp.pdf). The 2 fragments of the PARV4 capsid protein, aa 272–630 and aa 604–914 of ORF2, were fused to bacterial small ubiquitin-like modifier (SUMO) protein (a member of a ubiquitin-like protein family) and used as antigens in immunoblot. They were named viral protein (VP) 2 and VP3. For B19V, the antigens were VP1-specific (VP1-S; VP1, aa 1–227) fused to thioredoxin and VP2N (N terminal of B19V VP2, aa 1–343) fused to SUMO. The control protein was ribosomal P2 protein fused to SUMO. Antibodies to ribosomal P2 protein were rarely detected, except in patients with systemic lupus erythematosus (*5*).

Four of the 5 mothers had immunoglobulin (Ig) M against PARV4 (Table 2). Two of the 4 also had IgG against PARV4 (Figure, A, E); the other 2 had weakly positive IgM without IgG (not shown). No newborn had IgM against PARV4. We detected IgM against B19V in only 1 mother (Figure, A), who also had IgM against PARV4. None of the newborns had IgM against B19V. Two mothers (Figure, B, E) and 2 newborns had IgG against VP2N but not VP1S. The immunoblot pattern of IgG against B19V was inconsistent with findings in a previous report (*6*).

Only the mother and newborn of case A had detectable B19V DNA (genotype 1). By contrast, PARV4 DNA (genotype 2) was found in plasma of all but 1 of the 6 case-patients. The newborn negative for PARV4 DNA received a whole-blood exchange before sampling.

Conclusions

The first serologic study (*7*) and a recent study (*8*), both conducted in northern Europe, supported the fact that PARV4 is primarily a blood-borne virus. PARV4 DNA was detected in blood donors (*9*,*10*), and detection rates were 2% and 3.95%, respectively. The PARV4 seropositivity rate is expected to be higher than the DNA detection rate in blood donors because of the possibility of past infection (*2*,*11*,*12*). However, the IgG seroprevalence in 199 blood donors in France was 0%; the same rate was found in the general population in the United Kingdom (*13*). A much lower PARV4 DNA detection rate in blood donors in France may explain the result. Inconsistent with the findings of extremely low seroprevalence in France and the United Kingdom, PARV4 DNA was detected in the liver (15% and 41%, respectively) and the heart (41%)

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Table 1. Clinical information about 6 infants with hydrops, Taiwan, 2000–2009*

Patient	Sex	Delivery	Gestational age, wk	Birthweight, g	Hydrops signs	Hemoglobin, g/dL	Transfusion†	Platelets, 10^3/μL	Outcome
A	F	CS	35	2,846	Pericardial effusion, polyhydramnios	8.1	Yes	NA	Survived
B	M	Vaginal	40	2,468	Pleural effusion, skin edema	7.9	Yes	183	Survived
C	F	CS	32	3,070	Pleural effusion, skin edema	7.8	Yes	22	Died
D	M	CS	35	3,030	Ascites, pericardial effusion	4.1	Yes	6	Survived
E	M	Vaginal	27	1,450	Pleural effusion, skin edema	13	No	232	Survived
F	M	CS	32	2,634	Ascites, pleural effusion	12	No	76	Died

*CS, cesarean section; NA, not available.
†All transfusions were given after delivery. Blood samples were collected on the second (patients A–D) or third (E, F) day after delivery.

of non–HIV-infected patients in Germany (*11*) and Italy (*14*). PARV4 infection might be more widespread in some countries in Europe.

Contrary to the epidemiology of PARV4 in Europe, studies in Africa found different transmission routes and a higher seropositive rate in blood donors and the general population. In Ghana, 8.6% of infants had PARV4 viremia (*15*). In sub-Saharan Africa, 20%–37% of adults studied had antibodies to PARV4 (*13*). The groups studied in both reports did not have parenteral risk.

PARV4 can be transmitted through nonparenteral routes (*13*,*15*). Our study showed that placental transmission is one of them. PARV4 was unlikely to have been transmitted through a blood transfusion because of the low detection rate of PARV4 DNA in the blood donors. Because Taiwan has a high PARV4 seroprevalence rate (*4*), the possibility of a higher PARV4 DNA detection rate in blood donors is of concern. However, considering that the PARV4 seropositivity rate was 76.8% in HIV-infected intravenous drug users but only 6 of 350 had detectable DNA (*4*), the concern is not realistic.

Maternal PARV4 infections were diagnosed by detection of PARV4 DNA in all 5 mothers; 4 of whom had IgM against PARV4. Using IgM against PARV4 as evidence of recent infection must be done cautiously because of persistent IgM against PARV4 (*4*). Two mothers had weak IgM but no IgG against PARV4. The possibility of nonspecific IgM binding is low because of PARV4 viremia. The IgM result may be negative if the 2 samples are tested by enzyme immunoassay. The 2 mothers might have defective humoral immunity against PARV4 because we had detected 4 non–HIV-infected patients who had persistent IgM against PARV4 but did not have (or had weakly positive) IgG against PARV4 over 9–35 months. In a mother without IgM against PARV4, the amount of IgM might rapidly decline or a relapse of viremia might occur. In our previous longitudinal study of blood with IgM against PARV4, we found PARV4 DNA transiently during

Table 2. Antibody to B19V and PARV4 and detection of viral DNA in mothers and newborns, Taiwan, 2000–2009*

Case and patient	B19V		PARV4	
	Antibody	DNA	Antibody	DNA
A				
Mother	IgM+, IgG+; anti-VP1 and 2	+	IgM+, IgG+	+
Newborn	IgM–, IgG+; anti-VP1 and 2	+	IgM–, IgG+	+
B				
Mother	IgM–, IgG+ to VP2 only	–	IgM–, IgG+	+
Newborn	IgM–, IgG+; to VP2 only	–	IgM–, IgG+	+
C				
Mother	IgM–, IgG–	–	IgM weak +, IgG–	+
Newborn	IgM–, IgG–	–	IgM–, IgG–	–
D				
Mother	IgM–, IgG–	–	IgM weakly positive, IgG–	+
Newborn	IgM–, IgG–	–	IgM–, IgG–	+
E				
Mother	IgM weakly positive, IgG+, to VP2 only	–	IgM+, IgG+	+
F				
Newborn	IgM–, IgG+; to VP2 only	–	IgM–, IgG+	+

*B19V, parvovirus B19 virus; PARV4, parvovirus 4; Ig, immunoglobulin; +, positive; VP, viral protein; –, negative.

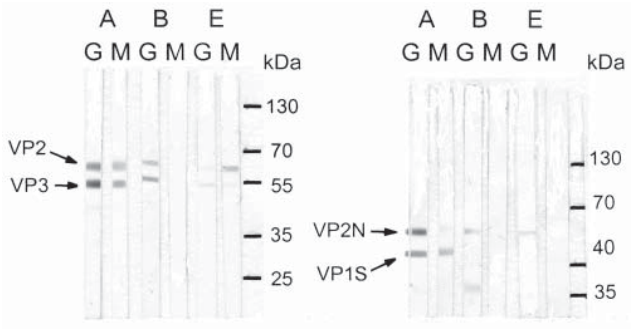

Figure. Immunoglobulin (Ig) G and IgM immunoblots of 3 mothers for infection with parvovirus 4 (PARV4) (left) or parvovirus B19 (B19V) (right). Case-patient A was co-infected with PARV4 and B19V; case-patient B was the only mother who did not have IgM against PARV4; case-patient E had weak IgM against PARV4 viral protein (VP) 3 and IgM against B19V VP2N, which could not be seen after scanning. Molecular weights are ≈60 kDa for PARV4 VP2, 51 kDa for PARV4 VP3, 51 kDa for B19V VP2N, and 41 kDa for B19V VP1S.

follow-up in 1 case (*4*). The mother without IgM against PARV4 was pregnant again 2 years later, and fetal death occurred at 18 weeks' gestation.

Persons with past B19V infection are expected to have IgG against B19V VP1 but not VP2 in immunoblot (*6*). On the contrary, 4 samples in this study had IgG against VP2N but not VP1S. We excluded the possibility of a reaction with SUMO protein by testing with the control protein. We tested 32 samples that had IgG against PARV4 and B19V VP2N using a commercial IgG B19V enzyme immunoassay (IBL, Hamburg Germany); 9 tested positive by IBL, and 8 were definitely positive because IgG against VP2N and VP1S were positive in our B19V immunoblot. Twenty-four samples had IgG against VP2N but not VP1S, only one of which tested positive by IBL. The paradoxical result was not seen in 47 blood samples without IgG against PARV4. Therefore, the best explanation is that PARV4 antibodies can cross-react with those of B19V VP2N.

In conclusion, PARV4 can be transmitted parenterally and placentally. Other transmission routes might exist and remain to be discovered. Prospective studies of PARV4 infection during pregnancy are needed to clarify the effect of PARV4 infection on fetal outcome.

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Similarity of Shiga Toxin–producing *Escherichia coli* O104:H4 Strains from Italy and Germany

To the Editor: Since the beginning of May 2011, a large outbreak of infections associated with Shiga toxin (Stx)–producing *Escherichia coli* (STEC) O104:H4 has occurred in Germany (*1*). The outbreak showed 3 unusual features: 1) a large proportion of case-patients with hemolytic uremic syndrome (HUS); 2) HUS in adults, although it usually affects children; and 3) frequent development of neurologic symptoms in patients when clinical and laboratory markers of HUS were improving (*1*,*2*). A second point-source outbreak caused by the same STEC O104 strain was reported in June 2011 in France (*3*). Both outbreaks were linked to eating fenugreek sprouts obtained from seeds produced in Egypt and distributed in Germany and other European countries (*4*).

Instead of the attaching–effacing mechanism of adhesion to intestinal mucosa that is typical of STEC associated with severe human disease (*5*), the STEC O104 epidemic strain had genetic markers and an adhesion pattern (*6*) typical of enteroaggregative *E. coli* (EAEC), another group of diarrheagenic strains found frequently in developing countries (*5*).

On basis of these findings, we reviewed our culture collection and found that an STEC strain (ED-703) from a case-patient with HUS in 2009 in Italy had the same combination of virulence factors as the strain from Germany: Stx2 production and enteroaggregative adhesion genetic markers. This strain, which had not been typed when it was isolated, showed positive PCR results for O104 (*7*) and H4 (*8*) antigen–associated genes and was agglutinated by an O104 antiserum (Statens Serum Institut, Copenhagen, Denmark). Pulsed-field gel electrophoresis showed a high degree of similarity (94.7%) with the outbreak strain from Germany (provided by M. Mielke, Robert Koch Institute, Berlin, Germany). In contrast with the outbreak strain, ED-703 did not produce extended-spectrum β-lactamases.

The strain from our culture collection had been isolated from a 9-year-old girl admitted to the pediatric nephrology unit of the Ospedale Maggiore (Milan, Italy) on August 5, 2009, after 5 days of bloody diarrhea, vomiting, and abdominal pain. Diagnosis of HUS was based on the presence of hemolytic anemia, thrombocytopenia, and anuria. Neurologic symptoms (e.g., lethargy, diplopia, and nystagmus) occurred during hospitalization; magnetic resonance imaging showed signal abnormalities in the lenticular nuclei.

Because of severe cardiac impairment with ejection fraction reduction and troponin increase, inotropic support and mechanical ventilation were temporarily needed. After improvement of clinical conditions, the patient was discharged, but she was readmitted a few days later because of headache, vomiting, confusion, dysarthria, hypertension, and visual impairment. Ischemic lesions were found by magnetic resonance imaging at fundus oculi. Neurologic status improved the next day, but the visual deficit persisted. Hemodialysis was needed for 2 months. Long-term sequelae of the disease were stage IV chronic kidney disease, hypertension, and severe visual impairment.

Informed consent and an epidemiologic interview were obtained from the patient's parents. The household, including her mother and 2 siblings (4 and 5 years of age), had traveled for 1 week to a resort in Tunisia; they had returned 3 weeks before the onset of the prodromal symptoms of HUS. Four days after their return, the youngest sister was hospitalized for 3 days because of bloody diarrhea, but no laboratory diagnosis was established. The mother reported having had watery diarrhea and abdominal pain on August 2. The patient history did not show any other usual risk factor for STEC infection, such as consumption of unpasteurized milk or dairy products, undercooked meat, or raw sprouts or direct exposure to ruminants or their manure. This finding suggests that the infection was probably acquired through person-to-person transmission.

This case report confirms that strains of STEC O104 strictly related to the epidemic strain in Germany had already caused sporadic infections in Europe (*9*). Other cases have been documented in 2001 in Germany (*6*,*9*), in 2004 in France (*9*), and in 2010 in Finland in a patient with diarrhea who had traveled to Egypt (*9*). Both of the cases for which the information on the origin of the infection was available were related to travel to northern Africa, from which the seeds associated with both outbreaks could be traced (*4*).

The history of this patient supports the hypothesis that ruminants would not have had a specific role in the transmission of STEC O104:H4, as already suggested by the epidemiologic features of the recent outbreaks (*1*,*3*). In fact, STEC O104 cannot be considered true STEC but rather EAEC strains that acquired the Stx2-coding phages by horizontal gene transfer, and EAEC is considered to be a human pathogen usually transmitted by the oral–fecal route (*5*).

The clinical course of our patient closely resembles those of persons who had HUS associated with the German outbreak (*1*,*2*). The unusual combination of virulence factors of STEC and EAEC, already described in a group of STEC O111:H2 from an outbreak of HUS in France in 1996

(*10*), might confer a high degree of virulence to these strains. It also might explain the severity of the clinical findings associated with STEC O104:H4 infections.

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Complicated Pandemic (H1N1) 2009 during Pregnancy, Taiwan

To the Editor: Pregnant women with pandemic (H1N1) 2009 virus infection are at increased risk for severe illness and complications (*1*–*3*). Recent reports have shown that this infection causes disproportionate illness and death in pregnant women and has been associated with adverse fetal and neonatal outcomes. We characterized the severity of pandemic (H1N1) 2009 virus infection among pregnant women in Taiwan.

Complicated influenza infection, defined as influenza-like illness and evidence of pneumonia, neurologic symptoms, myopericarditis, or invasive bacterial infections, has been a notifiable disease in Taiwan since 2002 (*4*). We reviewed reports and medical records of complicated pandemic (H1N1) 2009 virus infection, confirmed by real-time reverse transcription PCR in women 15–49 years of age who had onset of illness during July 1–December 31, 2009. Data were obtained for demographics; pregnancy status and outcome; gestational age at illness onset; preexisting medical conditions; onset of illness; treatment; and severity, including intensive care unit (ICU) admission.

To calculate rates of complicated pandemic (H1N1) 2009 virus infection, we estimated the pregnant population during July 1–December 31, 2009, by using the National Health Insurance computerized database for Taiwan (*5*). Women who were 15–49 years of age and had been assigned International Classification of Diseases, 9th Revision, Clinical Modification (www.cdc.gov/nchs/icd/icd9cm.htm), codes of V22* (normal pregnancy) and V23* (supervision of high-risk pregnancy) during the study were considered pregnant. Number of nonpregnant women was estimated by subtracting the calculated number of pregnant women from the number of women 15–49 years of age from 2009 household registration data (*6*). We estimated 95% confidence intervals (CIs) for rates by using exact binomial methods.

During July 1–December 31, 2009, data were reported for 10 pregnant women and 138 nonpregnant women 15–49 years of age who had confirmed, complicated pandemic (H1N1) 2009 virus infections. Dates of illness onset ranged from August 3 through December 31, 2009. Median age of the 10 pregnant women was 24.5 years (range 22–32 years), and median

gestational age at illness onset was 24 weeks (range 5–37 weeks). Other than pregnancy, none of these women had high-risk conditions for influenza complications recognized by the Advisory Committee on Immunization Practices (*7*). Seven women gave birth during hospitalization; 4 fetuses were stillborn, and 3 were live-born. At birth, the 3 live-born infants were at 27, 32, and 37 weeks' gestation and weighed 824, 1,850, and 3,270 g, respectively; all were admitted to a neonatal ICU.

Four (40%) pregnant and 84 (63%) nonpregnant women received oseltamivir treatment within 48 hours of illness onset (p = 0.19) (Table). Acute respiratory distress syndrome developed, mechanical ventilation was required, and extracorporeal membrane oxygenation was required in a higher proportion of pregnant women than nonpregnant women. Median length of hospital stay was 8 days (range 3–47 days) for pregnant women and 5 days (range 0–100 days) for nonpregnant women (p = 0.03). Five (50%) pregnant and 31 (22%) nonpregnant women were admitted to an ICU (p = 0.06); 3 (30%) pregnant women and 5 (4%) nonpregnant women died (p = 0.01).

There were 168,364 pregnant women and 6,220,197 nonpregnant women 15–49 years of age in Taiwan throughout the study period. The rate of complicated pandemic (H1N1) 2009 virus infection was 5.94 per 100,000 pregnant women (95% CI 2.85–10.92) and 2.22 per 100,000 nonpregnant women (95% CI, 1.86–2.62). Pregnant women were at greater risk for complicated pandemic (H1N1) 2009 virus infection than nonpregnant women (risk ratio 2.68, 95% CI 1.41–5.09).

Findings from this study have several limitations. Ascertainment of patients with complicated pandemic (H1N1) 2009 virus infection relied on passive surveillance. Therefore, data collection varied in completeness and quality between hospitals and different surveillance periods. The small number of pregnant women with confirmed complicated pandemic (H1N1) 2009 virus infection limited statistical power for stratified analyses by patient demographics and other characteristics. On November 1, 2009, Taiwan concurrently began a nationwide vaccination program against pandemic (H1N1) 2009 (*8*). As of December 31, 2009, a total of 8% of pregnant women and 13% of persons ≥15 years of age had been vaccinated (Taiwan Centers for Disease Control, unpub. data). Calculated rates of complicated pandemic (H1N1) 2009 virus infection could be affected by variable vaccine coverage among pregnant and nonpregnant women.

In Taiwan, oseltamivir treatment was provided free during the 2009 influenza pandemic to patients with influenza-like illness who had positive results for influenza by rapid influenza diagnostic tests, signs that signal progression to severe diseases (*9*), and clinical evidence of complicated influenza infections. The government recommended that pregnant women receive the vaccine against pandemic (H1N1) 2009, regardless of stage of pregnancy, and made this group a priority. Our findings are consistent with those of other studies (*1–3*) and suggest that pregnancy is a risk factor for severe or fatal pandemic (H1N1) 2009 virus infection in Taiwan. These findings justify policies to treat and vaccinate pregnant women against pandemic (H1N1) 2009.

Table. Characteristics of women ages 15–49 y who had confirmed pandemic (H1N1) 2009 infection, by pregnancy status, Taiwan, July 1–December 31, 2009*

Characteristic	Pregnant, n = 10	Nonpregnant, n = 138	p value†
Age, y	24.5 (22–32)	27.5 (15–49)	0.39
ACIP high-risk condition other than pregnancy	0	28 (20)	0.21
Pneumonia	9 (90)	134 (97)	0.30
ARDS	5 (50)	14 (10)	0.003
Admission to hospital	9 (90)	138 (100)	0.07
Time from symptom onset to hospitalization, d	2 (0–7)	2 (1–12)	0.78
Length of hospital stay, d	8 (3–47)	5 (0–100)	0.03
Admission to ICU	5 (50)	31 (22)	0.06
Length of ICU stay, d	16 (6–33)	5 (0–83)	0.07
Oseltamivir treatment	9 (90)	135 (98)	0.25
≤48 h after illness onset	4 (40)	84‡ (63)	0.19
Mechanical ventilation	5 (50)	19 (14)	0.01
ECMO	3 (30)	1 (1)	<0.001
Death	3 (30)	5 (4)	0.01
Time from illness onset to death, d	16 (2–37)	9 (1–32)	0.57

*Values are median (range) or no. (%). ACIP, Advisory Committee on Immunization Practices; ARDS, acute respiratory distress syndrome; ICU, intensive care unit; ECMO, extracorporeal membrane oxygenation.
†Medians were compared by using Wilcoxon rank-sum test, and proportions were compared by using Fisher exact test.
‡For 4 nonpregnant women, information on date of initiation of oseltamivir treatment was unknown.

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Pandemic (H1N1) 2009 and Seasonal Influenza A (H3N2) in Children's Hospital, Australia

To the Editor: We read with interest the report by Carcione et al. of clinical features of pandemic influenza A (H1N1) 2009 and comparison of these with 2009 seasonal influenza infection in a population-based study from Western Australia (*1*). Here we share our experience of hospitalizations for influenza in a tertiary care children's hospital in Sydney, New South Wales, Australia, during the 3 peak influenza seasons of the last decade.

During the 2009 Southern Hemisphere single influenza wave (June–September), we prospectively studied every child <15 years of age who was hospitalized with laboratory-confirmed influenza (74% had proven pandemic [H1N1] 2009) in Children's Hospital at Westmead (CHW), Sydney, as part of a collaboration between the National Centre for Immunisation Research and Surveillance and the Australian Paediatric Surveillance Unit. The study was approved by the Human Research Ethics Committee at CHW and supported by the state (New South Wales) health department. Data from hospitalizations for seasonal influenza at CHW in 2003 and 2007 (previous peaks in the last decade) were analyzed by using our previous studies and medical records (*2–4*). To compare pneumonia rates, we used the same case definitions in 2007 and 2009 (radiologic changes consistent with pneumonia). Proportions were compared by using the χ^2 statistic.

In 2009, the numbers of children with laboratory-confirmed influenza admitted to the hospital and to the pediatric intensive care unit (PICU) at CHW (226 and 22, respectively) were nearly double those admitted in 2007 (122 and 12) but similar to the number in 2003 (257 and 22). The proportion of case-patients admitted to the PICU, the length of hospital stay, and the length of PICU stay were similar in 2003, 2007, and 2009.

In 2009, among the 226 influenza-associated hospitalizations, 167 (74%) were for pandemic (H1N1) 2009 infection; in 2007, 119 of 122 influenza-associated hospitalizations were for seasonal influenza A (H3N2) infection (Table). During the 2009 pandemic wave, of all children admitted with laboratory-confirmed influenza, the proportion hospitalized with pandemic (H1N1) 2009 who were <6 months of age was similar to the proportion of children <6 months of age hospitalized with seasonal (H3N2) influenza in 2007 (21 [13%] of 167 and 21 [18%] of 119, respectively; p = 0.31). The proportions of those ≥5 years of age were significantly higher (61 [37%] and 15 [13%]; p = 0.0001). However, the proportion of those ≥5 years of age admitted to PICU in 2009 was less than in 2007 (10 [16%] of 61 vs. 3 [20%] of 15; p = 0.71). Similar percentages of children with preexisting conditions were admitted in 2009 and 2007 (47% and 49%, respectively). However, pneumonia was a more frequent complication in 2009 than in 2007 (42 [25%] of 167 vs. 15 [13%] of 119; p = 0.01). In 2009, the proportion of children with pandemic (H1N1) 2009 who needed mechanical ventilation (7 [4%] of 167) was similar to the proportion in 2007 who had seasonal influenza (H3N2) (6 [5%] of 119; p = 0.77). Furthermore, no child at CHW in 2007 or in 2009 received extracorporeal membrane oxygenation.

Vomiting occurred much more frequently in 2009 than in 2007 (59 [35%] of 167 vs. 16 [13%] of 119; p = 0.0001). In 2009, of 62 children who did not exhibit vomiting when first examined, and who were subsequently treated with antiviral drugs, only 1 had vomiting develop in the hospital. This condition resolved within hours, and

Table. Comparison of influenza-related hospitalizations, Children's Hospital at Westmead, Sydney, Australia, 2003, 2007, and 2009 influenza seasons*

Characteristics†	2009, pandemic (H1N1) 2009, no. (%), n = 167	Seasonal influenza A (H3N2), no. (%)	
		2007, n = 119	2003, n = 257‡
PICU admissions	18 (10.8)	12 (10.1)	22 (8.6)
Fatal cases (within 30 d of hospital admission)	0	0	3 (1.2)
Ventilated	7 (4.2)	6 (5.0)	14 (5.4)
Treated with antiviral drug§	92 (55.1)	16 (13.4)	0
Symptoms			
Vomiting§	59 (35.3)	16 (13.4)	NA
Diarrhea	21 (12.6)	8 (6.7)	NA
Seizure	11 (6.6)	7 (5.9)	NA
Complications			
Any complication	65 (38.9)	35 (29.4)	NA
Pneumonia¶	42 (25.1)	15 (12.6)	NA
Encephalopathy	5 (3.0)	2 (1.7)	NA
Preexisting condition	78 (46.7)	60 (50.4)	NA

*PICU, pediatric intensive care unit; NA, not available.
†Length of hospital stay, mean (range), d: pandemic (H1N1) 2009, 5.9 (1–107); seasonal influenza in 2007, 4.1 (1–50); seasonal influenza in 2003, 4 (1–28). Length of PICU stay, mean (range), d: pandemic (H1N1) 2009, 3.7 (1–30); seasonal influenza in 2007, 4.3 (1–25); seasonal influenza in 2003, 3.3 (0.6–11.4) Data for length of PICU stay in 2003 exclude 3 deaths; 1.3, 2.3, and 4.8 d after PICU admission (2.8 d mean PICU stay).
‡Only isolates from the 22 PICU case-patients were subtyped; all were H3N2.
§p = 0.0001.
¶p = 0.0104.

the 5-day course of antiviral treatment was completed. Chart review showed that in no child did antiviral treatment exacerbate vomiting, and no children required antiemetic treatment or intravenous rehydration. These data suggest that oseltamivir was uncommonly associated with vomiting in hospitalized children.

In macaque monkeys, pandemic (H1N1) 2009 virus is more pathogenic than seasonal influenza A (H1N1), particularly affecting the lungs (*5*). The significantly higher proportion (and number) of pneumonia patients at CHW in 2009 than in 2007 (Table) seems to provide additional evidence of the pathogenecity of pandemic (H1N1) 2009 virus. However, the number of children with laboratory-confirmed influenza admitted to the hospital was similar in 2009 and 2003. Given that the sensitivity of influenza laboratory tests has improved over time (e.g., greater use of nucleic acid tests), these data suggest that the incidence of hospitalization during the 2009 pandemic was not greater than the incidence during the 2003 influenza season.

Although pneumonia appeared more likely to be diagnosed in hospitalized children in 2009 than in 2007, we observed no increase in the risk for serious outcomes (PICU admission or ventilation rate, length of admission, or death) in children hospitalized with pandemic (H1N1) 2009 infection than in children hospitalized with seasonal influenza (H3N2) during the 2 peak years studied. The 2009 pandemic had an effect on children's services that was comparable to the busiest interpandemic influenza season of the previous decade. The large number of admissions and complications, including in children with no existing medical condition, supports the need for universal influenza vaccination.

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Global Health Security in an Era of Global Health Threats

To the Editor: Global health security is the protection of the health of persons and societies worldwide. It includes access to medicines, vaccines, and health care, as well as reductions in collective vulnerabilities to global public health events that have the potential to spread across borders. For example, transboundary zoonotic diseases such as avian influenza (H5N1) infections affect animals and humans, thereby threatening health security worldwide because of their high death rates (≈60% in humans) (*1*).

During the past 15 years, fairly standardized responses to threats have been implemented around the globe. Some of these responses have been against severe acute respiratory syndrome and avian influenza (H5N1), which have been overseen by a well-resourced international health system (*2*).

These global health threats have raised the highest levels of political and social concern. This concern has provoked governments and international agencies to address health threats through a security rationale, which emphasizes the themes of national security, biosecurity, and human security. This amalgamation of health issues and security concerns has produced a notion of health security, which is dominated by technical medical approaches and pharmaceutical interventions. These approaches and interventions have already begun to shape the way international health policy is formulated (*3*).

A global vision of health security is very much part of contemporary rhetoric. However, this vision lacks the drive and speed needed to make proposals materialize and operationalize ideas in the geographic areas where they are most desperately needed. Small benefits accrue to members of vulnerable populations who, in fact, are those most likely to be affected by epidemic diseases. A public health security design that impinges on a global approach runs the risk of neglecting cultural, economic, ecologic, and social conditions on the ground. Regional approaches that address hazards and threats may be more inclusive of context-specific conditions (*4*).

Global public health threats related to infectious pathogens of animal origin are expected to rise. To address these threats, several experts and strategists suggest the initiation of a worldwide early-alerting and -reporting mechanism. Aggregation of disease threats through an event-focused Web-based platform could enable this mechanism. This timely gathering of disease intelligence can inform policymakers about the nature of risks. Disease maps can display details needed to design tailored policies and control measures to tackle diseases according to their specifics (*5*).

Leading scientists and researchers continue to try to understand the global temporal and spatial patterns of animal diseases. This understanding is gained through an array of instruments, ranging from the use of satellite images to cutting-edge molecular technologies. The momentum so far has created an open forum for decision-makers to collaborate with the leading international agencies to advocate for surveillance, identification, and control of zoonotic diseases to uphold global public health security (*6*).

However, global initiatives suffer from the free-rider problem and from moral hazards. Some low-income countries with weak governance have alerted the international community about their fragile health care systems to capture a nontrivial portion of funds that seldom reach their intended destinations. These resource allocations to developing countries foster aid dependence (*7*).

The international technical agencies tasked with upholding animal and human health should remain at the forefront of identifying and addressing evolving threats. This process will demand continuous flexibility, agility, and a coordinated international effort. Attaining goals of mitigating threats and reducing risks posed by the emergence of zoonoses requires close collaborations with national health authorities and local governments. The large investments planned to improve foresight and prevention might or might not work. If they do not work, apportioning blame to countries or regions for disease flare-ups can result in social, political, cultural, and economic consequences that in the past have turned out to be unjustified, unfair, and ultimately detrimental (*8*).

Clearly, global health threats can be reduced only by the concerted

actions of national and international actors. In the years ahead, the international community will almost certainly be expected to bring its formidable technical knowledge, skills, and analytic capabilities to confront this expanded global health threat environment (*9*).

It would be wrong, however, to forget the many insights that current advances in epidemiology and surveillance have delivered. In fact, should the impetus to finance a global health agenda encounter opposition or obstacles, it would seem easier and logical to strengthen already functional activities.

Lastly, the realities and the prevalent policymaking environment have created a trap between a desire to prioritize global health by portraying aspects of it as an existential security issue and the fact that security ultimately might not be the most useful language for describing and institutionalizing the health threats and hazards confronted by societies around the world (*10*). Regardless of whether a trap has been created, action is urgently needed.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Use of Workplace Absenteeism Surveillance Data for Outbreak Detection

To the Editor: We applaud Mann et al. on their use of a school-based absenteeism surveillance system to compare daily all-causes absenteeism data against a historic baseline to detect outbreaks of influenza-like illness (ILI) as an adjunct to traditional disease reporting (*1*). The growing availability of electronic human resources systems has increased the potential to harness near real-time workplace absenteeism data to complement school absenteeism surveillance and other sources of traditional outbreak surveillance.

In London, United Kingdom, during the first wave of pandemic influenza A (H1N1) 2009, workplace absenteeism data from the Transport for London attendance/absence reporting system were compared with the historical baseline 3-year mean for comparative weeks of the year. The proportion of Transport for London employees absent because of self-reported or medically certified ILI, during June 28–October 17, 2010, generated surveillance alerts when compared with historical baseline data above the 95th and 99th percentile thresholds (SDs 1.96 and 2.58). For the same period, cause-specific workplace influenza absenteeism data were highly correlated with routinely published ILI surveillance, including the National Pandemic Flu Surveillance and sentinel General Practitioner systems (Figure) (*2*).

In Australia, workplace all-causes absenteeism for a major Australia-wide employer has been included as a nonspecific indicator of influenza surveillance by the Australian government for >15 years. A recent study during a severe influenza season in Australia confirmed that employee

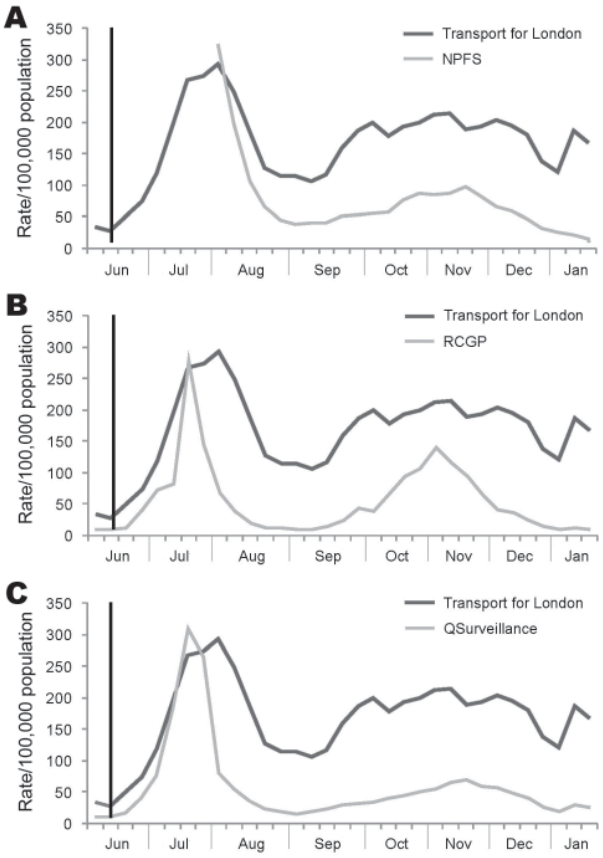

Figure. Comparison of transport for London absenteeism rates from influenza data to syndromic surveillance indicators of influenza-like illness rates, London, United Kingdom, 2009. A) National Pandemic Flu Service (NPFS); B) Royal College of General Practitioners (RCGP); and C) QSurveillance. Vertical black line indicates when the World Health Organization declared a pandemic on June 11, 2009. Source: Health Protection Agency, London, and Transport for London.

absenteeism was highly correlated with laboratory-confirmed influenza, and such information could be used to provide surveillance alerts up to 2 weeks before other traditional influenza surveillance data sources (*3*).

The use of workplace absenteeism data, particularly from large employers, has the potential for overcoming the major limitation of school-based absenteeism data in detecting outbreaks of ILI: the effects of school holidays and local planned school closures. Near real-time workplace absenteeism is an effective surveillance tool and should be more widely incorporated in influenza surveillance systems.

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Zoonotic Ascariasis, United Kingdom

To the Editor: *Ascaris lumbricoides/suum* is a complex of closely related enteric roundworms that mainly infect humans and pigs (*1*). Transmission occurs through ingestion of fecally excreted ova. *A. lumbricoides* worms usually infect humans, mainly in regions with poor sanitation, where the environment is contaminated with human feces. In industrialized countries, human ascariasis is uncommon and cases are generally believed to have been imported (*2*). By contrast, *A. suum* infection of pigs occurs worldwide; in the United Kingdom, 3.4%–6.5% of pigs at slaughter have evidence of infection (*3*). Sporadic zoonotic infection with *A. suum* in the industrialized world is described (*4*–*6*) but poorly quantified. We describe probable zoonotic transmission of *Ascaris* spp. roundworms in Cornwall, a rural county in southwestern England.

Incidence rates for ascariasis in Cornwall and the rest of England were calculated from local and national laboratory data. From 2004 through 2008, a total of 18 cases were

identified in Cornwall, and 314 from the rest of England were reported to the Health Protection Agency; annual rates were 0.87 and 0.12 cases per 100,000 population, respectively.

From 1995 through 2010, a total of 63 ascariasis cases were identified in Cornwall, and details of patient age, sex, and place of residence were collected. Patients from Cornwall were younger (mean age 22 years) than those from other parts of England (mean age 31 years), and the proportion of patients <5 years of age in Cornwall (35.5%) was greater than that in the rest of England (19.7%). Similar proportions (61% vs. 65%) of patients from Cornwall and England were female.

The possibility of zoonotic transmission in Cornwall was investigated by comparing risk factors for ascariasis and enterobiasis (caused by an enteric helminth that infects only humans). From 1995 through 2010, the laboratory in Cornwall identified 38 cases of *Enterobius* infection. Patient mean age was 24 years (range 1–95 years); 2 (5.7%) patients were <5 years of age and 23 (60.5%) were female. The following risk factors were considered for statistical analysis: age <5 years, female sex, and residence near pig herds. Residence was determined by comparing the postcodes of case-patients with postcodes of pig holdings registered with the Department of Environment, Food and Rural Affairs. The UK postal service allots a maximum of 80 households to a postcode. In rural areas like Cornwall, the number is much smaller. Consequently, sharing a postcode with a pig holding implies proximity to pig herds. Of the 50 ascariasis patients with a Cornwall postcode, 11 (22%) shared that postcode with a pig holding. Of the 35 enterobiasis patients in Cornwall, only 2 (5.7%) shared a postcode with a pig holding.

Odds ratios were calculated for all 3 risk factors, and the Fisher exact test was used to determine their significance. We calculated p values by using 2-tailed models for age and sex and a 1-tailed model to test the association with residence near a pig holding (Table). Significant associations were found for age <5 years (odds ratio 6.42, p = 0.0037) and living near pigs (odds ratio 4.65, p = 0.036) but not for female sex.

Further evidence for zoonotic transmission comes from molecular analyses of DNA extracted from 11 *Ascaris* spp. worms recovered from patients in Cornwall. Results were compared by PCR-linked restriction fragment length polymorphism and sequence analysis with those from 35 reference worms from pigs in the United Kingdom, Denmark, Uganda, Guatemala, and the Philippines and from 20 worms from humans in Uganda, Tanzania, and Nepal. We used the PCR-linked restriction fragment length polymorphism method described by Nejsum et al. (*5*). Briefly, the ribosomal internal transcribed spacer region was amplified, and the products were digested with the restriction enzyme *Hae*III and separated into bands by agarose gel electrophoresis. All worms from humans and pigs in the United Kingdom had 3- or 4-banded genotypes, typically found in worms from pigs (*4,5,7*). By contrast, a 2-banded genotype predominated in worms collected from humans living in *A. lumbricoides*–endemic areas. Similarly, sequence analysis, as described by Nejsum et al. (*8*), of amplified mitochondrial *cox*1 genes using primers by Peng et al. (*9*) showed that all worms from humans in Cornwall clustered with worms from pigs (i.e., had pig-like DNA sequences).

Compared with the rest of the United Kingdom, incidence of human ascariasis is high in Cornwall, especially among children <5 years of age. Because of the retrospective nature of our study, we have little travel or clinical information for these case-patients. However, because such young case-patients would probably not travel much and because postcode data identified place of residence as a risk factor, the data suggest a focus of locally acquired *A. suum* infection in humans in Cornwall. Molecular evidence implicates pigs as the source. Further studies are needed, but if pigs are confirmed to be the source, control and prevention of this emerging infection will probably depend more on modifications of animal husbandry and fecal waste disposal rather than on human sanitation.

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Table. Risk factors for ascariasis versus enterobiasis in residents of Cornwall, United Kingdom, 1995–2010

Risk factor	Ascariasis, no. with risk factor/total no. cases*	Enterobiasis, no. with risk factor/total no. cases†	Odds ratio (95% confidence interval)	p value‡
Age <5 y	22/62	3/38	6.42 (1.77–23.30)	**0.0037**
Female sex	35/62	22/38	0.845 (0.372–1.920)	0.83
Pig holding within same postcode	11/50	2/35	4.65 (0.962–22.500)	**0.036**

*n = 63 patients. Postcode not available for 12 patients; 1 postcode was outside of Cornwall. Thus, only 50/63 patients were included in postcode analysis. Sex not indicated for 1 patient; age not indicated for 1 patient. Thus, only 62/63 patients were included in age and sex analyses.
†n = 38 patients. Postcode outside of Cornwall for 3 patients. Thus, only 35/38 patients were included in postcode analysis.
‡By Fisher exact test. **Boldface** indicates significance.

Migrant Health Section Health Protection Agency, Centre for Infections, London, UK, for providing laboratory notification data; and Animal Health, Department for Environment, Food and Rural Affairs, Worcester, UK, for providing pig-holding registrations.

The article is dedicated to the memory of Carole Fitzsimons, who was instrumental in planning this study and collecting the worms, but who died before the project was complete.

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Minority K65R Variants and Early Failure of Antiretroviral Therapy in HIV-1–infected Eritrean Immigrant

To the Editor: Genotypic drug resistance testing before initiation of first-line antiretroviral therapy (ART) is recommended to detect drug-resistant viruses and to avoid treatment failure caused by preexisting drug-resistant viruses (*1*). However, standard resistance testing cannot detect drug-resistant HIV-1 minority variants unless they represent 20%–25% of the population (*2*). Approximately 15% of those who underwent seroconversion in the acute phase in industrialized settings harbor drug-resistant HIV-1 minority variants, while standard resistance testing did not detect drug-resistant viruses in those patients (*3*). We report the case of a treatment-naive HIV-1–infected patient with early treatment failure because of preexisting minority K65R-harboring HIV-1 variants.

A 32-year-old immigrant to Switzerland from Eritrea with a recently diagnosed HIV-1 subtype C infection was seen at University Hospital, Zurich. On the basis of the low CD4+ T-cell count of 69 cells/μL (15%) and high HIV-1 viral load of 980,000 copies/mL plasma, we started directly observed ART with tenofovir and emtricitabine plus nevirapine. Genotypic resistance testing showed no evidence of resistance. Within the first 4 weeks of ART, the viral load decreased to 540; however, 4 weeks later it increased to 15,000, and then 12 days later to 71,000 HIV-1 RNA copies/mL (Figure). Resistance testing at this time revealed the reverse transcriptase (RT) mutations K65R, K103N, and M184V, which confer resistance to all prescribed drugs. ART was changed to lamivudine/zidovudine, darunavir/ritonavir, and etravirine, and subsequently viremia decreased and remained undetectable.

We hypothesized that preexisting drug-resistant HIV-1 minority variants might have caused this early treatment failure. Thus, we performed clonal analysis of the RT gene before and during ART. At baseline, 17/222 clones (7.7%) carried the K65R mutation, synonymous to ≈51,000 HIV-1 RNA copies. Later, the K65R mutation was comprised in all clones. Further preexisting drug-resistance mutations have been detected in single, K65 wild-type, separate clones: K70R, V106A, and V108I. However, neither the K103N nor the M184V mutation was detected in any of those 222 clones, but both mutations were rapidly selected during early treatment failure.

Thus, the presence of the K65R mutation in a substantial fraction

of the virus population and the rapid acquisition of the K103N and M184V mutations led eventually to early treatment failure. We cannot formally rule out that K103N mutants were present before ART at a very low frequency <0.5% and that K65R/K103N double mutants were potentially selected. The M184V mutation was acquired later because only 12.5% of K65R/K103N viruses carried the M184V mutation at the first time point during treatment failure.

Because of the considerable absolute number of less replication-competent K65R-harboring viruses in the patient, we assume that this variant has been transmitted. The additional presence of isolated, nonnucleoside RT inhibitor (NNRTI)/nucleoside RT inhibitor (NRTI) resistance mutations is another indication for the transmission of drug-resistant viruses, although they and the K65R mutation were not found in the same viral genomes. Presumably, the index patient was treated with nevirapine, stavudine, and lamivudine, the commonly prescribed first-line ART in resource-limited settings (*4*).

The prevalence of K65R-harboring drug-resistant HIV-1 minority variants is not negligible in treatment-naive patients. We have shown that 2.7% of HIV-1–infected patients, mainly those infected with HIV-1 subtype B, harbor the K65R mutation as a minority variant (*5*), which is comparable with the prevalence of minority K65R-harboring variants (4%) in patients from South Africa who are infected with HIV-1 subtype C (*6*). A meta-analysis showed that limited or unavailable HIV-1 RNA monitoring in combination with ART regimens with a low genetic barrier to resistance in resource-limited settings is associated with high rates of NNRTI/NRTI resistance in patients for whom ART fails (*7*). Although ritonavir-boosted protease inhibitors containing ART regimens have a higher genetic barrier to resistance and would be preferable in such settings, they are generally not part of first-line therapy in resource-limited settings. Thus, proper monitoring and drug-resistance testing would be desirable when using NNRTI-based ART regimens. Moreover, it could be beneficial to apply sensitive assays for the detection of drug-resistant HIV-1 minority variants in clinical practice. However, clinical cut-off levels for those minority variants need to be defined.

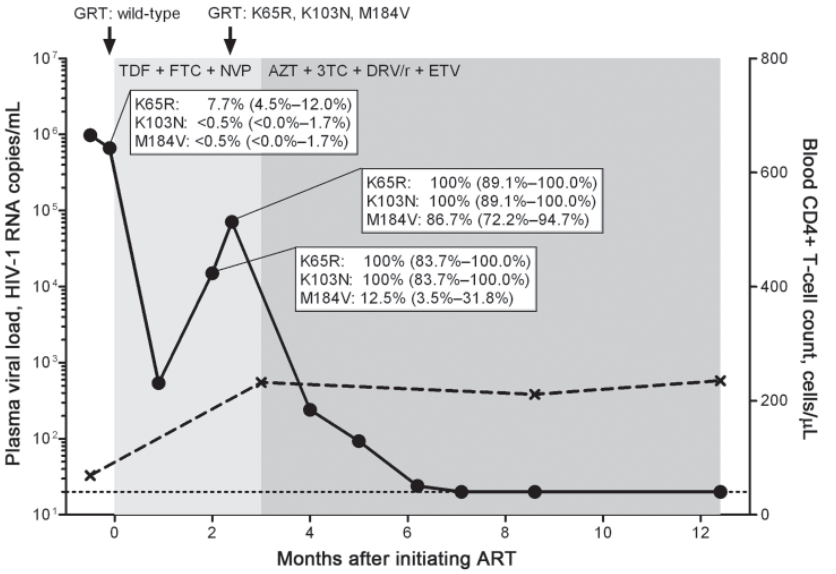

Figure. Kinetics of viremia, CD4+ T-cell count, and drug resistance mutations in a treatment-naive person from Eritrea, infected with HIV-1 subtype C, who was experiencing early antiretroviral therapy (ART) failure. Viral load (circles) was measured by using the Cobas AmpliPrep TaqMan HIV-1 test version 2.0 (Roche Diagnostics, Rotkreuz, Switzerland) with a detection limit of 20 HIV-1 RNA copies/mL plasma (dotted line). CD4+ T-cell count is depicted in crosses. Genotypic resistance testing (GRT) based on population sequencing was performed at time points indicated by black arrows. Duration of different ART regimens is shown in shades of gray. Part of the HIV-1 reverse transcriptase (codons 52–218) gene was cloned and sequenced at time points before ART and during virologic failure: n = 222 clones at –2 days before ART, n = 24 clones, and n = 38 clones during virologic failure, respectively. The dynamics of the selection of the K65R, K103N, and M184V mutations are depicted by percentages and 95% confidence intervals. TDF, tenofovir; FTC, emtricitabine; NVP, nevirapine; AZT, zidovudine; 3TC, lamivudine; DRV/r, darunavir in combination with ritonavir; ETV, etravirine.

Immigration from resource-limited settings is increasing, and data suggest that minority variants harboring the K65R mutation are quite prevalent in those infected with HIV-1 subtype C, who are treatment naive (*6*). The case described here demonstrates that these drug-resistant HIV-1 minority variants can quickly accumulate further drug-resistance mutations and lead to early treatment failure, especially in the context of an ART regimen with low genetic barriers to resistance. This case was the first among immigrants treated in our center, but, given the potential for considerable transmission rates of resistance in countries that lack virologic monitoring, this mutation could become a larger problem.

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Diagnosis of Rickettsioses from Eschar Swab Samples, Algeria

To the Editor: Tick-borne rickettsioses are zoonoses caused by intracellular bacteria belonging to the spotted fever group rickettsiae (*1*). The main clinical signs are high fever, maculopapular rash, and an inoculation eschar at the site of the tick bite (corresponding to the portal of entry of rickettsiae into the host). Recently, several rickettsioses were diagnosed by using swab samples from skin lesions (*2*,*3*). In an animal model, as long as eschars were present, rickettsial DNA was detected (*2*). Our aim was to evaluate the advantage of skin swab samples for diagnosis of rickettsial diseases in a country where rickettsioses are endemic (*4*).

From July 2009 through October 2010, a total of 39 patients in the infectious disease department of Oran Teaching Hospital, Algeria (27 men, 12 women; median age 46.5 years) were included in a prospective study. The mean (± SD) interval between onset of lesions and consultation was 7 ± 1.8 days. Underlying conditions were present in 13 (33%) patients: diabetes (4 patients, 10.2%), hypertension (2 patients, 5%), chronic renal failure (1 patient, 2.5%), cervical cancer (1 patient, 2.5%), bronchial cancer (1 patient, 2.5%), and tobacco consumption (6 patients, 15%). Fever and generalized maculopapular rash (also on palms and soles) were found for 38 (97.4%) patients, including 5 (12.8%) with purpuric rash. One (2.5%) patient had 2 eschars on the back. Eschars were frontal (1 [2.5%] patient), cervical (3 [7%]), axillary (4 [10.2%]), on the back (6 [15%]), on the abdomen (2 [5%)], on the thorax (1 [2.5%]), inguinal (6 [15%]), on the leg (8 [20.5%]), on the arm (1 [2.5%]), on the buttock (2 [5%]), on the breast (1 [2.5%]), on the nipple (1 [2.5%]), on the penis (1 [2.5%]), and on the scrotum (2 [5%]). Conjunctivitis was reported for 24 (61.5%) patients and myalgia for 34 (87.8%). Lymphadenopathy was found near the eschar for 8 (20.5%) patients. Antimicrobial drug therapy (doxycycline 200 mg 1×/d) for 3.5 ± 1.4 days was empirically prescribed for all case-patients before diagnoses were confirmed.

A dry sterile swab (Copan, Brescia, Italy) sample was collected from the inoculation eschar of each patient by the same person (N.M.). Two patients had 2 swab samples collected from the same eschar. Only 1 swab sample was collected from the patient with 2 eschars. Scabs were removed from eschars before swabbing. The swabs, while being rotated vigorously, were directed to the base of the eschar at a 50°–60° angle for 5–6 times. For 4 patients, an eschar biopsy sample was collected under sterile conditions. Swabs were then placed back in their tubes and stored at –20°C before transportation to Unité des Rickettsies, Marseille, France.

In the laboratory, each sample was placed in 2 mL of culture medium. DNA was extracted from

Table. Acceptability of swab or biopsy samples from tick-bite eschars, Oran Teaching Hospital, Oran, Algeria, July 2009–October 2010

Question	Answer, no. (%)*			
	Eschar swab sample	Skin biopsy sample	Both samples	Nothing
For medical or biologist practitioners, n = 78				
In the case that your patient has a "tache noire," what would you do to confirm the diagnosis?	46 (59)	5 (6.4)	22 (28)	5 (6.4)
If you have a "tache noire," what do you do to confirm the diagnosis?	57 (73)	2 (2.5)	9 (11.5)	9 (11.5)
For patients, n = 58				
In the case of inoculation eschar, which kind of medical procedure would you prefer your medical doctor to conduct?	43 (74)	78 (12)	5 (8.6)	3 (5.2)

*p = 0.0001 for eschar swab sample vs. skin biopsy sample; p value not available for other parameters.

200 μL of solution of eschar swab or skin biopsy samples by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, with a final elution volume of 100 μL. We used quantitative real-time PCR (qPCR) to determine quality of DNA extraction and level of housekeeping gene coding for β-actin (*5*) and to detect rickettsiae (*2*,*5*). Mean (± SD) cycle threshold (C_t) value of the β-actin gene for all swab samples was 24.9 (± 2.7). Of 41 swab samples, 26 were positive for rickettsial DNA (63.4%) by qPCR targeting the RC0338 gene (*5*); mean C_t value was 33.99 (± 2.15). Specific *Rickettsia conorii conorii* qPCR, targeting the putative acetyltransferase gene (*2*), had positive results for 25 (64%) patients; C_t values ranged from 31.02 to 38.63. This sensitivity is comparable to that of PCRs for detecting *R. conorii*, the agent of Mediterranean spotted fever, on skin biopsy samples (*4*,*6*). Mean C_t value for β-actin gene amplification of *R. conorii conorii*–positive swab samples was 23.84 (± 2.19), significantly lower than that for *R. conorii conorii*–negative samples (26.84 ± 2.72; p = 0.0003). Of 4 patients for whom swab and skin biopsy samples were available, 3 had positive results.

Opinions of health care providers and patients were evaluated by using standard questionnaires (Table). Most health professionals preferred collecting swab samples over biopsy samples for patients and for themselves (46 vs. 5 and 57 vs. 2, respectively; p = 0.0001). Patients from France and Algeria also preferred having a swab sample taken over a skin biopsy sample (43 vs. 7; p = 0.0001). Statistical analyses were conducted by using GraphPadPrism version 2.0 (www.graphpad.com/prism/Prism.htm) to p≤0.5.

Swabbing an eschar is a rapid and simple technique that can be easily performed without risk for the side effects associated with biopsy sampling. Insufficient material taken during swabbing, evidenced by high C_t values of β-actin, results in low rickettsial load, explaining the false-negative results. This test can be used at the bedside or in an outpatient clinic and could be useful for epidemiologic and clinical studies. Because qPCR results can be obtained <4 hours after sampling, this technique might be useful for point-of-care diagnosis.

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Livestock-associated Methicillin-Susceptible *Staphylococcus aureus* ST398 Infection in Woman, Colombia

To the Editor: *Staphylococcus aureus* causes health care– and community-associated infections worldwide in humans and animals. It also asymptomatically colonizes a large proportion (20%–60%) of otherwise healthy individuals. In recent years, various countries have reported an increasing number of humans infected with livestock-associated *S. aureus* multilocus sequence type (ST) 398, which suggests that this strain is emerging in community and health care settings (*1*). Methicillin-resistant *S. aureus* (MRSA) ST398 has received particular attention as a causative agent of infection in pigs, dogs, horses, cattle, and poultry. Colonization and infection in humans have also been described in Europe (*2*), Asia (*3*), Canada (*4*), and the United States (*5*), particularly among persons with frequent exposure to animals, such as farmers, veterinarians, and their household members. However, infections with MRSA ST398 and methicillin-susceptible *S. aureus* (MSSA) ST398 have recently been described in persons with no history of contact with livestock (*6*–*10*).

We report infection of a woman with MSSA ST398 in Colombia, South America. On November 3, 2009, this 82-year-old woman was admitted to the emergency unit of the Hospital Universitario San Vicente Fundación in Medellín, reporting a 15-day history of fever, dyspnea, and pain in her left leg. She lived in a rural area and reported previous contact with dogs and chickens. Her medical history included diabetes mellitus, hypertension, valvular heart disease, and chronic arterial occlusive disease. Four months earlier she had received a femoro–popliteal vascular prosthetic graft in her left leg.

At the time of admission, blood culture was requested, and intravenous vancomycin (1 g every 12 hours) and piperacillin/tazobactam (4.5 g every 8 hours) were empirically administered. *S. aureus* was subsequently isolated from blood culture, and antimicrobial drug susceptibility was assessed in accordance with Clinical Laboratory Standards Institute guidelines by using a Vitek 2 instrument (bioMérieux, Marcy l'Etoile, France). The isolate was susceptible to methicillin, rifampin, and vancomycin but resistant to clindamycin, erythromycin, gentamicin, levofloxacin, minocycline, moxifloxacin, tetracycline, and trimethoprim/sulfamethoxazole. Additional laboratory results showed an elevated leukocyte count with predominant polynuclear neutrophils and increased C-reactive protein levels (21.2 mg/L).

Angiography of the left femoro–popliteal segment showed a collection surrounding the entire vascular prosthetic graft, which was presumed to be the bacteremic focus. Accordingly, rifampin (600 mg every 12 hours) was added to the regimen, the femoro-popliteal graft was surgically removed, the collection was drained, and the limb was amputated. After the surgery, cephradine was administered for 14 days, after which clinical signs and symptoms of bacteremia resolved completely, and the patient was discharged from the hospital.

The blood culture isolate was subsequently confirmed as *S. aureus* by PCR with primers directed to the *nuc* gene. Genes encoding the following virulence factors were also evaluated by PCR, but none were detected: Panton-Valentine leukocidin, arginine catabolic mobile element, staphylococcal enterotoxins A–E, exfoliating toxins A and B, and toxic shock syndrome toxin 1. Genotypic analysis indicated that the isolate belonged to multilocus ST398 (allelic profile 3-35-19-2-20-26-39) and *spa* type t571 (eGenomics *spa* type 109); pulsed-field gel electrophoresis with *Sma*I digestion yielded no results, as described previously for ST398 (*1*).

This report documents the emergence of human infection caused by MSSA *spa* type t571 ST398 in South America. Despite being about only 1 case, this report nevertheless highlights the changing epidemiology of *S. aureus* within the region. The study was limited by the inability to sample animals from a surrounding farm to determine the potential for zoonotic spread of *S. aureus* in domestic environments. Notably, *spa* type t571 ST398 has been found recently in MSSA carriage isolates from New York City (*6*), the Dominican Republic (*6*), and the Amazonian region of French Guiana (*9*) and in clinical MSSA isolates from the Netherlands (*7*), People's Republic of China (*8*), and France (*10*). Given the patients' absence of contact with livestock in most of these reports, transmission of MSSA ST398 *spa* type t571 may not be limited to animal exposure, suggesting the possibility of person-to-person spread. Accordingly, our finding reinforces the need to heighten awareness of the transmission and virulence potential of MSSA ST398, particularly in developing countries where understanding of *S. aureus* colonization and transmission dynamics is probably limited. Such information has implications for the design of appropriate control measures to reduce human and animal infections from this emerging pathogen.

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Granulicatella adiacens and Early-Onset Sepsis in Neonate

To the Editor: *Granulicatella* and *Abiotrophia,* formerly known as nutritionally variant streptococci, are normal flora of the human upper respiratory, gastrointestinal, and urogenital tracts (*1*). *G. adiacens* has been associated with bacteremia and endovascular, central nervous system, ocular, oral, bone and joint, and genitourinary infections (*1–4*).

Although streptococci are a frequent cause of early-onset sepsis in newborns, non–group B or D streptococci comprise only ≈1% of cases of early-onset neonatal sepsis; the condition is primarily associated with viridans streptococci (*5*). This report describes a male infant with early-onset sepsis caused by *G. adiacens*. Molecular genetic studies identified the same organism in flora isolated from the maternal cervix, which suggests vertical transmission.

After 36 weeks' gestation, a male infant, weighing 2,850 g, was born by repeat caesarean section to a 37-year-old woman who was negative for group B streptococcus; she began labor without rupture of membranes. Apgar scores were 9 and 9 at 1 and 5 minutes, respectively. Respiratory distress developed in the infant within an hour of birth. Peripheral blood and cerebrospinal fluid (CSF) samples were obtained, and intravenous ampicillin (150 mg/kg every 12 h) and gentamicin (4 mg/kg every 24 h) were administered. Leukocyte count was 27,000/mm^3 with 79% polymorphonuclear leukocytes and 2% band forms; platelet count was 223,000/mm^3. CSF cell counts were 3 leukocytes/mm^3 and 18 erythrocytes/mm^3.

Respiratory distress progressed rapidly, and at 20 hours of life, mechanical ventilation was instituted. Chest radiograph demonstrated diffuse, bilateral interstitial infiltrates consistent with pneumonia. Persistent pulmonary hypertension was diagnosed by echocardiography. Peripheral blood culture yielded *G. adiacens* (API 20 STREP, bioMérieux Clinical Diagnostics, Durham, NC, USA) that was sensitive to vancomycin. Repeat blood samples were obtained before and after antimicrobial drug treatment was changed to vancomycin, 10 mg/kg every 12 h, and gentamicin, 4 mg/kg every 24 h. CSF culture and repeat blood cultures had no growth. Vancomycin and gentamicin were administered for 14 days. The patient eventually recovered and was discharged after 25 days of hospitalization. The biochemical identification of *G. adiacens* in the blood culture was confirmed by 16S rRNA gene sequencing.

Cervical samples for culturing were obtained from the mother 4 days after delivery. After samples were plated on blood and chocolate agar, colonies were harvested from the primary plates, and pooled bacterial DNA was isolated for further analyses. A primer pair was designed to amplify a 422-bp fragment unique to *G. adiacens*. These primers (sense, 5′-GGTTTATCCTTAGAAAG GAGGT-3′, and antisense, 5′-GAGC ATTCGGTTGGGCACTCTAG-3′) were used to amplify *G. adiacens* from DNA prepared from the bacterial pools isolated from maternal cervix and, as control, from DNA prepared from the infant's blood culture isolate. DNA amplicons underwent agarose gel electrophoresis with ethidium bromide staining and were visualized under ultraviolet light. PCR amplification showed DNA amplicons for the patient's blood culture, his mother's cervical swab sample, and a positive control but not for negative controls (Figure). Capillary nucleotide DNA sequence analysis confirmed all 3 amplicons as *G. adiacens* (not shown).

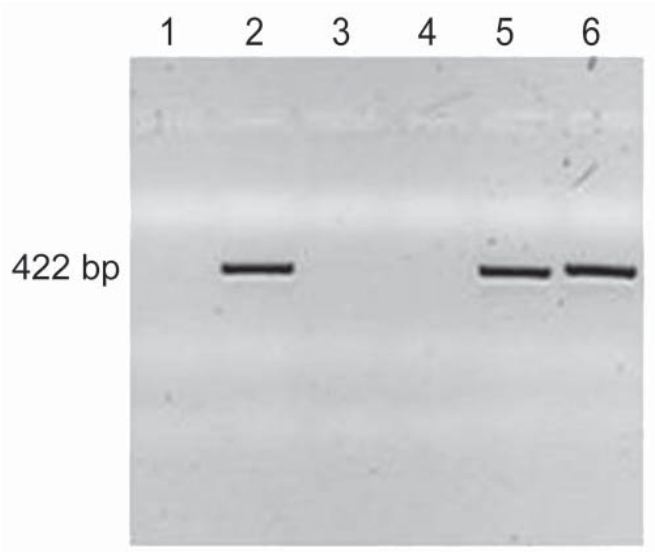

Figure. PCR amplification of *Granulicatella adiacens* DNA. DNA was prepared from the bacteria indicated, PCR-amplified with *G. adiacens*–specific primers, and subjected to agarose gel electrophoresis with ethidium bromide staining and ultraviolet light visualization. Lane 1, water, negative control; lane 2, *G. adiacens*–positive control, ATCC 49175; lane 3, DH5α *Escherichia coli*–negative control (Invitrogen, Carlsbad, CA, USA) 18263–012; lane 4, *Enterobacter sakazakii*–negative control, ATCC BAA-894 (American Type Culture Collection, Manassas, VA, USA); lane 5, patient's blood culture isolate; lane 6, DNA isolated from a pool of bacteria obtained from the cervix of the patient's mother. DNA amplicons were amplified only from the positive control, the patient's blood culture, and his mother's cervical swab specimen. Sequence analysis confirmed all 3 amplicons as *G. adiacens.*

Nutritionally-variant streptococci have been difficult to identify because they have unique culture requirements, they exhibit pleomorphic phenotypic features (*1*), and they demonstrate satellitism around colonies of other bacteria. This has led to misidentification or nonidentification by using commercially available phenotypic testing, prompting the recommendation that 16S rRNA gene sequencing be used for *G. adiacens* identification (*6*).

The Clinical and Laboratory Standards Institute does not recommend disk-diffusion testing to determine the susceptibility of *G. adiacens* to antimicrobial drugs and suggests broth microdilution MIC testing in cation-adjusted Mueller-Hinton broth with 2.5%–5% lysed horse blood and 0.001% pyridoxine hydrochloride (*7*,*8*). Under these conditions, 55% of *G. adiacens* isolates were susceptible to penicillin, 63% were susceptible to ceftriaxone, 96% were susceptible to meropenem, and 100% were susceptible to vancomycin (*4*). A more recent report indicates that the resistance of *G. adiacens* to β-lactam and macrolide antimicrobial drugs may be increasing (*3*).

Treatment of *G. adiacens* infection may be difficult. In vitro antimicrobial drug susceptibility patterns do not correlate well with clinical response to treatment, and *G. adiacens* infections often respond poorly to antimicrobial drug treatment. Higher rates of bacteriologic failure and relapse rates after treatment have been observed for *G. adiacens* infection than for infections with streptococci and other related genera (*9*). *G. adiacens* endocarditis has been associated with a more severe clinical course than endocarditis caused by enterococci or viridans group streptococci, with higher rates of illness and death. On the basis of studies demonstrating synergy in vitro and in vivo, empiric combination therapy with vancomycin and an aminoglycoside has been suggested until susceptibility data are available (*10*), particularly when treating serious conditions such as endocarditis (*3*).

Reports have increased of serious infections caused by *Granulicatella* and *Abiotrophia,* including this report of early onset neonatal sepsis due to *G. adiacens*. One recent study described 8 isolates associated with invasive infection in a 4-year period (*3*). Increased awareness and identification of *G. adiacens* are likely contributing to this increased recognition, with earlier cases not detected or diagnosed. 16S rRNA gene sequencing may facilitate or confirm *Granulicatella* and *Abiotrophia* spp. as the etiologic agents of infection.

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Lymphocytic Choriomeningitis with Severe Manifestations, Missouri, USA

To the Editor: Lymphocytic choriomeningitis virus (LCMV) is an arenavirus maintained zoonotically in house mice (*Mus musculus*) and may also be carried by pet rodents, especially hamsters (*1–3*). Infection of healthy humans usually results in nonspecific febrile illness. However, LCMV infection can cause severe symptoms, including aseptic meningitis (*4*).

Early data suggested ≤8% of central nervous system manifestations of viral etiology were caused by LCMV (*5*). In contrast, in a more recent study of 91 cases of encephalitis among persons with potential rodent contact, LCMV was not detected (*6*). We describe 2 recent unrelated LCMV infections with central nervous system manifestations, which were associated with rodent exposures, as a reminder that LCMV should be considered in cases of aseptic meningitis of unknown etiology.

In July 2008, an 89-year-old man in Missouri, USA, with a history of hypertension received a prescription for metolazone and was given methotrexate (2.5 mg orally, 3×/wk) because of a pharmacy error. Two weeks later, he showed confusion, speech difficulty, and had a fever. When hospitalized 3 weeks after symptom onset, the patient was drowsy but able to answer questions and had a supple neck, plantar responses in extension, and a temperature of 38.8°C. Methotrexate treatment was then stopped.

Routine laboratory test results were within reference ranges, with the exception of a serum aspartate aminotransferase level of 92 U/L (reference range 0–37 U/L) and an alanine aminotransferase level of 78 U/L (reference range 0–65 U/L). Two blood cultures were sterile, a chest radiograph showed cardiomegaly, and cranial computed tomography without contrast showed moderate cerebral atrophy.

The patient was empirically given intravenous ceftriaxone (1 g) and intravenous azithromycin (500 mg, 1×/d for 7 days). Two days after he was hospitalized, cranial magnetic resonance imaging showed mild-to-moderate, chronic, small vessel ischemia and involutional changes. Serologic test results for HIV and West Nile virus were negative. On the fourth hospital day, lumbar puncture yielded clear, colorless cerebrospinal fluid (CSF) containing 1 erythrocyte and 98 leukocytes/high-powered field (1% neutrophils, 95% lymphocytes, 4% monocytes); protein level was 127 mg/dL (reference range 15–45 mg/dL), and glucose level was 40 mg/dL (reference range 43–70 mg/dL). CSF test results for bacteria, cryptococcal antigen, and herpes simplex virus (HSV) and PCR result for *Borrelia burgdorferi* were negative. CSF and serum submitted to the Centers for Disease Control and Prevention showed LCMV-specific

immunoglobulin (Ig) M titers (serum 6,400, CSF 1,280); IgG was not detected. Virus was not isolated.

The patient's son-in-law reported that mice had been trapped in the patient's home during the previous winter. The patient was treated supportively and discharged from the hospital 30 days after admission. Six weeks after discharge, the patient was well, without residual neurologic or cognitive deficits.

In November, 2010, a 34-year-old woman in Missouri was hospitalized with a 1-day history of progressive headache, neck pain, photophobia, nausea, and vomiting. She had a history of asthma and migraine headaches and had twice undergone surgical repair of congenital heart defects in childhood. She was alert and cooperative, her neck was supple, results of a neurologic examination were normal, and her temperature was 38.6°C.

Laboratory studies showed a hemoglobin level of 11.0 g/dL, a hematocrit of 33.1%, a peripheral leukocyte count of 9,600 cells/mm^3, a platelet count of 308,000/mm^3, a serum creatinine level of 0.7 mg/dL, and normal liver functions. A chest radiograph showed mild interstitial changes. Cranial computed tomography without contrast showed normal results. Lumbar puncture showed hazy, colorless CSF, no erythrocytes, 544 leukocytes/high-powered field (11% neutrophils, 84% lymphocytes, 5% monocytes), a protein level of 122 mg/dL, and a glucose level of 56 mg/dL. Results of Gram staining; bacterial culture; and tests for enterovirus, HSV-1, HSV-2 (PCR), and West Nile virus (IgM and IgG) were negative.

The patient was empirically given a 7-day course of ceftriaxone (2 g intravenously 1×/d) and vancomycin (1 g intravenously every 12 hours). CSF submitted to the Centers for Disease Control and Prevention was positive for LCMV by PCR and virus isolation. LCMV-specific IgM (titer 1,280) was detected; IgG was not detected. The patient had observed mice in her home 3 months before hospitalization, although she did not recall any direct contact with them. Her condition improved with supportive therapy and she was released.

These patient observations underscore the potential for severe manifestations of LCMV infection. Neurologic manifestations may mimic those of infections with other viruses, including HSV-1, HSV-2, enteroviruses, and arboviruses, necessitating specific diagnostic tests for identification of the cause, particularly in patients who report recent contact with wild or pet rodents.

The ubiquitous nature of the LCMV reservoir and documented infections in numerous localities in the United States and internationally imply widespread geographic risk for LCMV infection (*7*). Serosurveys in the southern and eastern United States have indicated previous infection in ≈3%–5% of persons (*1*,*8*). However, recent data from upstate New York indicated a seroprevalence <1% (*9*). Thus, the risk for LCMV infection can be minimized by active exclusion and trapping of rodents in the home and avoidance of pet rodents.

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Sporotrichosis Caused by *Sporothrix mexicana*, Portugal

To the Editor: Sporotrichosis is a subcutaneous fungal infection present worldwide that is caused by traumatic inoculation or inhalation of spores of the dimorphic fungus *Sporothrix schenckii* complex (*1*–*3*). However, molecular studies have shown that the *S. schenckii* complex constitutes several cryptic infectious species (i.e., *S. albicans, S. brasiliensis, S. globosa, S. luriei, S. mexicana*, and *S. schenckii*). Marimon et al. (*4*) demonstrated 3 major clades grouped into 6 putative phylogenetic species. The natural habitats of these species are soil and plants. The species showed distinct pathologic behavior, antifungal responses, and phenotypes, which suggests that optimal clinical treatment may depend on the taxon involved in the sporotrichosis (*1*). Human infections have been reported primarily from the Americas, including Latin America (*3*,*5*). Asia (e.g., Malaysia, India, Japan), Africa, and Australia are also regions where infections are endemic (*6*). Although infections are rare in Europe, a case of human infection (*7*) and a case of an animal infection (*8*) have been described in southern Italy. We report a case of human sporotrichosis in which *S. mexicana* was isolated from a patient in Portugal.

A 34-year-old man sought care at a podiatry clinic in Vila Nova de Famalicão, Portugal, in 2009 for multiple polymorphous eruptions and ulcers on both feet. There was no obvious cause of the disease. Although the patient had traveled to Malaysia in 2003 and had worn open footwear every day, he did not recall receiving a skin wound. In 2004 in Portugal, subcutaneous nodules appeared in both feet, became ulcerated, and spontaneously healed. By 2005, more severe lesions had appeared and became a chronic infection in both feet and lower limbs. The symptoms were diagnosed erroneously as dyshidrotic eczema, and treatment with topical corticosteroids was unsuccessful.

Several skin fragments of the lesions were submitted for mycological assessment. Fungi were not found on potassium hydroxide slides of all samples. Filamentous fungal colonies were observed after 7 days of culture on Sabouraud dextrose agar slopes at 25°C. The fungus had hyaline septate hyphae, with hyaline and dematiaceous conidia compatible with *Sporothrix* spp. The isolate was accessed and preserved in the Micoteca da Universidade do Minho (MUM, Braga, Portugal) fungal culture collection and given the accession code MUM 11.02.

The macroscopic features and sporulation were analyzed by using cornmeal and potato dextrose agars. Clusters of intercalary or terminal conidia were formed by sympodial growth from differentiated conidiophores on both media. Sympodial conidia were hyaline or slightly pigmented. Sessile conidia were predominantly subglobose, obovoidal or ellipsoidal, and 3.35 ± 0.41 μm long by 2.30 ± 0.32 μm wide (Figure, panel A). A teleomorph was not observed. The colony diameter on potato dextrose agar after 21 days of incubation attained 40 mm at 30°C and 5 mm at 37°C. The yeast form was achieved by incubating the isolate on brain heart infusion agar on slants at 35°C ± 2°C for 7 days in a single subculture.

Dextrose, sucrose, and raffinose assimilation tests were performed in triplicate by using yeast nitrogen base medium. The strain assimilated dextrose, sucrose, and raffinose,

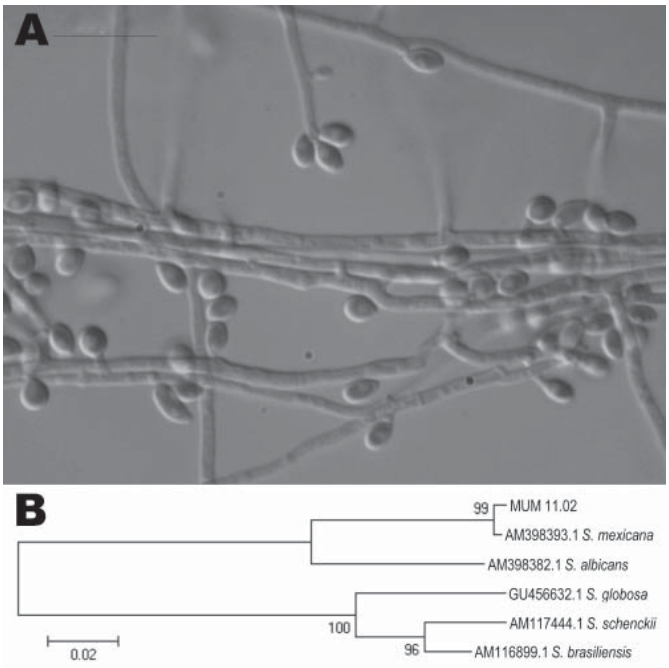

Figure. A) Photomicrograph of sympodial and sessile conidia of *Sporothrix mexicana* obtained by using a transmitted differential interference contrast microscope. The isolate was obtained from a patient in Portugal in 2009 and archived in the Micoteca da Universidade do Minho (MUM) under accession no. MUM 11.02. Scale bar = 10 μm. B) Neighbor-joining tree showing relatedness of MUM 11.02 isolate with other species of the *S. schenckii* complex. The percentage of replicate trees in which the associated taxon clustered in the bootstrap test (1,000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 537 positions in the final dataset. Scale bar indicates nucleotide substitutions per site.

showing phenotypic characteristics typical of *S. mexicana* and *S. schenckii* (*2*). In contrast, type reference strain *S. brasiliensis* CBS 120339 was included in the test, and it was able to assimilate only dextrose.

A presumptive identification based on phenotypic characteristics allowed us to classify this fungus as *S. mexicana*, although this species has an atypical morphologic profile. The diameter of colonies grown at 30°C and 37°C are smaller than those proposed by Marimon and collaborators but much closer to those of *S. schenckii* (*2*). These differences could be attributable to the intraspecific variation of this single isolate.

Genomic DNA was obtained from the yeast phase of *S. mexicana* MUM 11.02, and the partial sequencing of the nuclear calmodulin gene was based on the amplicon generated by PCR reaction by using CL1 and CL2A primers (*2*,*3*). Sequencing was performed at Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. A BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) comparing the sequence of the calmodulin gene with sequences AM398382, AM398393, AM117444, AM116899, and AM116908 in the GenBank database confirmed the identity of this isolate as *S. mexicana*. The MUM 11.02 isolate showed 99% similarity with the sequences of *S. mexicana* (i.e., GenBank accession no. AM398393) with high bootstrap support values (Figure, panel B). The calmodulin sequence of MUM 11.02 was deposited in GenBank as JF970258.

In vitro susceptibility tests with fluconazole, itraconazole, and terbinafine were performed by the microdilution method (*9*) and revealed MICs of 128 μg/mL, 32 μg/mL, and 0.5–1.0 μg/mL, respectively, which corresponds to the findings of Marimon et al. (*1*) for *S. mexicana*. Thus, *S. mexicana* is an emerging cause of human sporotrichosis.

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Swinepox Virus Outbreak, Brazil, 2011

To the Editor: *Swinepox virus* (SWPV), which replicates only in swine, belongs to the *Suipoxvirus* genus of the *Poxviridae* family. It is the etiologic agent of a skin disease of pigs, characterized by generalized pustular lesions and associated with high rates of illness (occasionally >80%). It occurs mainly on farms with poor management and housing conditions and affects primarily pigs <3 months of age; adult pigs show milder signs. The disease is mechanically transmitted by pig lice or through direct animal contact (*1*). *Vaccinia virus* (VACV; *Orthopoxvirus* genus) also causes a similar pustular disease in pigs that is difficult to distinguish clinically from SWPV infections. VACV infections were common during smallpox vaccination

campaigns, when VACV was transmitted to domestic animals from lesions of vaccinees (*1*,*2*).

Swinepox disease has a worldwide distribution, and 4 outbreaks of similar infections were reported in pig herds in Brazil during 1976–2001 (*3*). Nevertheless, the etiologic agents of these outbreaks have never been identified through molecular techniques. Specific virus identification in such infections is particularly relevant in Brazil, considering the persistence of VACV in nature in this country, causing frequent outbreaks of pustular skin disease in dairy cattle (*4*–*6*). Therefore, distinguishing between SWPV and VACV infections during outbreaks of pustular disease in pigs is essential for evaluating whether VACV infection might have spread to pigs and whether SWPV could be detected in Brazil.

We describe the molecular identification of SWPV as the etiologic agent of an outbreak of pustular disorder in pig herds. In November 2010 and January 2011, ≈850 of 3,460 animals on 3 pig farms in Holambra, São Paulo, Brazil, had generalized pustular lesions on the body, fever (38.0°C–39.7°C) and mild weight loss. Lesions evolved from macules or papules to umbilicated lesions with pustular content, followed by crusting (online Appendix Figure, www.cdc.gov/EID/content/17/10/11-0549-appF.htm). Secondary dermatitis was also noticed. Healing occurred after 3–4 weeks, but the disease started subsequently in previously healthy animals. Although the first clinical signs of disease started in the nursery units (pigs 40–50 days old), nearly 70% of the sick pigs were at the finishing units (pigs 127–134 days old), where elevated animal density and deficient sanitation conditions were observed. These findings may account for the high attack rate (nearly 50%) in finisher pigs, although overall illness was moderate (nearly 25%) when animals from all units were analyzed together. No deaths were associated with the outbreak, in concordance with the low death rates reported for SWPV infections (<5%) (*1*). The affected farms belonged to the same owner, who reported frequent movement of animals between the farms.

Scabs from 7 animals were used for DNA extraction (*4*), followed by PCR detection of poxvirus DNA (*7*). We used primers designed to anneal to gene regions conserved in different poxviruses: FP-A2L, 5′-TAGTTTCAGAACAAGGATA TG-3′ and RP-A2L, 5′-TTCCCATAT TAATTGATTACT-3′ directed the amplification of a 482-bp fragment of the virus late transcription factor–3 (www.poxvirus.org); primer sets for the DNA polymerase gene (543-bp fragment) and DNA topoisomerase gene (344-bp fragment) were previously described (*7*). Amplicons were directly sequenced as described (*4*,*5*). Consensus primers that specifically detect the full-length hemagglutinin gene of Eurasian-African orthopoxviruses were used to investigate VACV in the samples (*4*).

The nucleotide sequences obtained for the fragments of the DNA polymerase, DNA topoisomerase, and virus late transcription factor–3

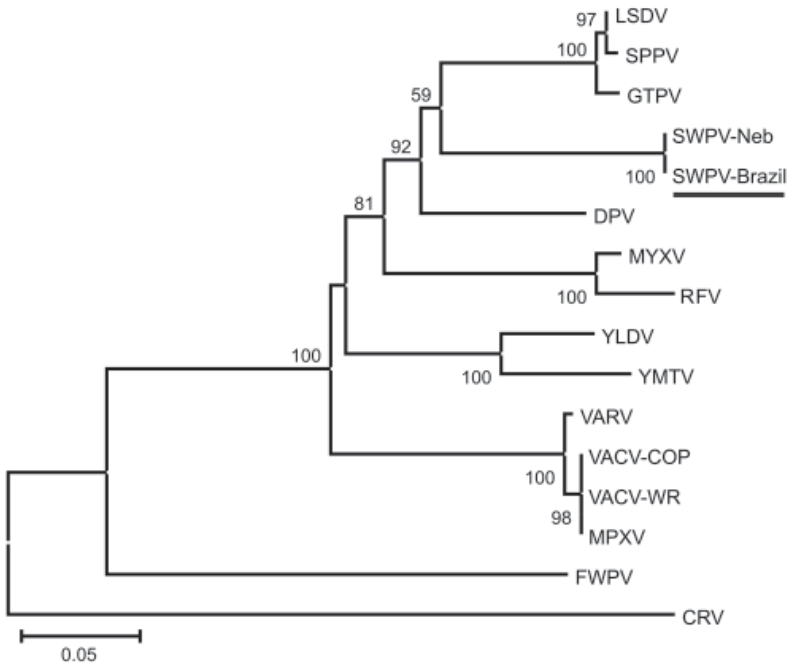

Figure. Phylogenetic tree based on the predicted amino acid sequences of fragments of the DNA polymerase, DNA topoisomerase, and viral late transcription factor-3 of the clinical isolate (GenBank accession nos. JF770341, JF770342, and JF770343) and 15 poxviruses. Sequences were aligned by ClustalX version 1.81 (www.clustal.org), and the concatenated alignments were used for phylogeny inference (MEGA4; www.megasoftware.net) opting for the neighbor-joining method and Poisson correction. We computed 1,500 replicates for bootstrap support. Values >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Virus species and GenBank accession numbers: LSDV (lumpy skin disease virus; AF409137), SSPV (sheeppox virus; AY077834), GTPV (goatpox virus; AY077834), SWPV-Neb (swinepox virus Nebraska strain; NC_003389), DPV (deerpox virus; AY689437), MYXV (myxoma virus; NC_001132), RFV (rabbit fibroma virus; NC_001266), YLDV (Yaba-like disease virus; NC_002642), YMTV (Yaba monkey tumor virus; NC_002642), VARV (variola virus; NC_002642), VACV-COP (vaccinia virus Copenhagen strain; M35027), VACV-WR (vaccinia virus WR strain; NC_006998), MPXV (monkeypox virus; DQ011154), FWPV (fowlpox virus; NC_002188), CRV (crocodilepox virus; NC_008030). Virus isolated in this study is underlined.

of the clinical specimens were aligned with sequences from other poxviruses available in the public database (GenBank). They showed 100% nt identity with their orthologs of SWPV Nebraska strain. Concatenated amino acid alignments were used for phylogenetic inference (Figure). The clinical isolates and SWPV branched together in the phylogenetic tree with high bootstrap support. No amplification of the hemagglutinin gene was obtained, demonstrating that the animals were not infected with VACV. Samples were also negative for *Erysipelothrix* spp. (by PCR and ELISA) and porcine circovirus-2 (by PCR).

Outbreaks of swinepox disorders have been frequently reported in Europe, North America, and Oceania, and special attention has been given to congenital cases, which usually lead to high case-fatality rates (*2*,*8*,*9*). Our data identified SWPV as the cause of a recent outbreak in Brazil and suggest that previous outbreaks in the neighboring municipality of Campinas in 1976 and 1980 (*3*) may have been caused by SWPV as well because pigs are the only host and reservoir of the virus. Further sequencing analysis of the virus isolates will be necessary to characterize the strain of SWPV circulating in Brazil.

Recently, an outbreak of VACV-related disease in horses was reported in southern Brazil, which alerted the scientific community to the possible spread of this disorder to animal hosts other than dairy cattle (*10*). However, our data clearly demonstrate that this outbreak in pigs does not represent a spread of VACV infection, despite frequent reports of VACV-related outbreaks in dairy cows in São Paulo State (*6*). Therefore, the differential diagnosis of skin diseases of pigs might be a useful tool in epidemiologic surveys to access VACV spread and host range in Brazil.

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Plasmodium vivax Seroprevalence in Bred Cynomolgus Monkeys, China[1]

To the Editor: Having worked with numerous species of research nonhuman primates over the past 26 years, I have a keen interest in related occupational health and safety. In this regard, I was quite interested in the recent report by Li et al. (*1*) and have some comments and questions relative to this article.

The occurrence of *Plasmodium* spp. infection in feral primates, feral source captive primates, or primates

[1]Li et al. have declined to respond to this letter.

bred outdoors in malaria-endemic areas is not uncommon. However, with the exception of *P. knowlesi*, it is my understanding that malarial organisms found in cynomolgus monkeys do not pose a major zoonotic concern (although this can always change). Furthermore, it is my understanding that *P. vivax* does not infect macaques, including cynomolgus monkeys.

Other malarial parasites of cynomolgus monkeys, apart from *P. knowlesi*, may include *P. cynomologi*, *P. inui*, *P. fieldi*, and *P. coatneyi*. A recent publication reported that in wild-source cynomolgus monkeys in Malaysia, >90% of the animals tested were positive for ≥1 *Plasmodium* species. Furthermore, >80% of samples from these animals were positive by specific PCR for ≥1 of these organisms (*2*).

Using PCR for *Plasmodium* spp. identification, I have tested newly imported research cynomolgus monkeys from various breeding centers in China. I can confirm that some animals have subclinical malarial infections.

Except for the report by Li et al. (*1*), I am unaware of other reports of *P. vivax* in cynomolgus monkeys. It would be interesting to confirm the presence of this organism by using PCR primers specific for *Plasmodium* spp. My questions to the authors relate to the test method used in their study. Was an ELISA for detecting *P. vivax* antibodies the only diagnostic method used to identify this parasite? It may be useful to re-address the specificity of this test in differentiating various *Plasmodium* spp. Until these issues are clearly addressed, their reported results are not reliable.

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Dengue Virus Serotype 4, Roraima State, Brazil

To the Editor: Temporão et al. recently reported the detection and characterization of dengue virus serotype 4 (DENV-4) in Boa Vista, Roraima, Brazil (*1*). To date, 4 subtypes of DENV-4 have been recognized: genotype I, which comprises Asian strains (e.g., Thailand-1978-U18441); genotype II, which has been detected since the early 1980s in the Americas (e.g., Brazil-1982-U18425); genotype III, which comprises recently emerged Thai strains (GenBank accession no. AY618989); and genotype IV, which comprises sylvatic strains (GenBank accession no. EF457906) (*2*).

Temporão et al. conducted phylogenetic analysis of envelope gene sequences and concluded that 3 samples of DENV from Roraima in 2010 were DENV-4, genotype I (*1*). Unfortunately, the authors mistakenly labeled Asian strains (Thailand-1978 and -1985) as genotype II, and American strains (e.g., Brazil-1982) as genotype I. Those DENV-4 strains isolated in Roraima in 2010 in fact belong to genotype II (*2*,*3*). We had previously analyzed 2 samples isolated from Roraima in 2010 by using C/prM nucleotide sequencing and maximum-likelihood phylogenetic reconstruction. Our results, presented at the XXI National Meeting of Brazilian Society for Virology in October 2010, show that both isolates are indeed genotype II (*3*). Nucleotide sequences are available in GenBank under accession nos. HQ822125 and HQ822126.

Temporão et al. also concluded that because only genotype II (reported as genotype I) was identified in their samples, "[it] excludes the possibility that Asian genotypes previously circulated in Brazil." Beyond its obviously flawed logic, we believe that this statement lacks scientific support; DENV-4 genotype I, closely related to Chinese and Philippine strains, has in fact been shown to occur in the city of Manaus, ≈800 km south of Boa Vista, as reported in 2 recent articles (*4*,*5*). Circulation of DENV-4 genotype I in northern Brazil, probably related to increasingly intense trade with Asian countries, may be sporadic and geographically limited as yet (*5*), but ignoring this evidence can hardly be helpful for dengue surveillance and control.

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In Response: With regard to their comment on our letter, Dengue Virus Serotype 4, Roraima State, Brazil (*1*), Amézaga Acosta et al. correctly refer to an erroneous correlation between genotype I and American strains and between genotype II and Asian strains (*2*). That was a typographic error in the text and figure. Our Figure shows the correct association.

Amézaga Acosta et al. also suggested a scientific inconsistency, that our results exclude circulation of previous Asian strains in Brazil, and argue that Figueiredo et al. (*3*) published a letter on the introduction of DENV-4 in Manaus. However, Amézaga Acosta et al. probably do not know that the article by Figueiredo et al. has been contested by the Brazilian Ministry of Health for failing to demonstrate any irrefutable scientific result, including the virus isolation. Our statement was logically based on strong epidemiologic surveillance, virus isolation, serologic evidence (hemagglutination inhibition assay and immunoglobulin ELISA), and clinical aspects.

Regarding circulation of 2 genotypes in Brazil, when the article was written, epidemiologic and molecular evidence supported the hypothesis of circulation of only the American genotype (II) in northern Brazil and not the Asian (I) and American (II) genotypes at the same time (*1,2,4*). More specifically to the Manaus finding, no virus was isolated and no strong serologic evidence (in the lack of virus isolation) was provided, and the Ministry of Health considers this article a mistake, probably caused

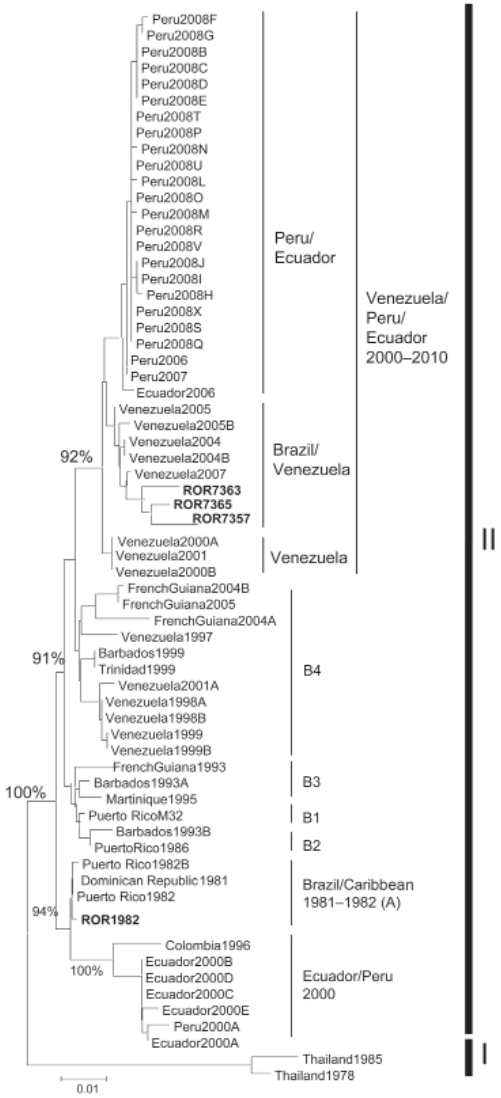

Figure. Phylogenetic tree demonstrating the 2 clusters of dengue virus serotype 4 correctly associated with corresponding genotypes: genotype II, American strains, including strains isolated in Roraima in 2010; and genotype I, 2 Asian strains. **Boldface** indicates strains isolated in Roraima. Scale bar indicates 1% nucleotide sequence divergence.

by laboratory contamination with Asian genotype I.

Possible introduction and detection of Asian DENV-4 strains in Brazil should not be ignored because the possibility of multiple introduction events in the country resulting from intense transit of people and commercial activities across Brazil from the Caribbean and Asian regions poses a real risk. However, at this time, only genotype II has been isolated and genetically characterized (*1*). The previously published articles lack strong and reliable scientific evidence.

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Novel Hepatitis E Virus Genotype in Norway Rats, Germany

To the Editor: We read with interest the article by Johne et al. about 2 novel hepatitis E virus (HEV) isolates in Norway rats in Germany (*1*). Some points in the report deserve comment.

First, because of degeneracy of the genetic code, HEV amino acid sequences are more conserved than nucleotide sequences. For instance, although the open reading frame 2 of the avian HEV isolate (GenBank accession no. AY535004) has only 65% nt sequence homology to that of the swine HEV isolate swGX32 (GenBank accession no. EU366959), their amino acid sequences shared >90% identity. However, the table in (*1*) indicated the amino acid sequence homologies between the novel and previous HEV isolates were similar to (some even lower than) the nucleotide sequence homologies. These low sequence identities of the capsid proteins between the novel and previous HEVs may explain why no HEV antibody–positive rat was found in the initial serologic screening with a commercial genotype 1–based ELISA. Furthermore, we wonder how the novel antigen in the hepatocytes could react with the anti-HEV serum in the immunohistochemical staining.

Second, the authors stated they determined the entire virus genome by using a previously described method (*2*). The primers in that method were designed to amplify a genotype 3 HEV isolate with low (55.7%) sequence homology to the 2 novel HEV isolates and therefore cannot amplify their sequences. We ask the authors to list the new primer sequences they used, which will help determine the full viral genome if this virus is found in other regions or animal species.

Suggesting the rabbit HEV sequences may be representative genotype 3 isolates is not yet appropriate because not enough research has yet determined whether rabbit HEV infects other species. Therefore, the rabbit HEV sequence FJ906895 should not be listed as representative genotype 3 isolate as in Figure 1 in (*1*). Also, the swine isolate DQ450072 should not be listed as a representative genotype 4 isolate; a recent report indicated it was a recombinant produced between genotypes 3 and 4 isolates (*3*).

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In Response: The major objective of our study was determination and initial characterization of the entire nucleotide sequence of a novel hepatitis E virus (HEV) from Norway rats. We demonstrated high levels of nucleotide and amino acid sequence divergence between HEV strains from the novel rat and other mammalian and avian HEV strains. In line with our data, nucleotide (and amino acid) sequence identities of 50%–53% (42%–49%), 51%–57% (42%–55%), and 45%–46% (20%–29%) were reported for open reading frame (ORF) 1, ORF-2, and ORF-3, respectively, between rat, human, and avian HEV strains (*1*). Of course, the genetic code is degenerated; however, no strict relationship exists between divergence in nucleotide and corresponding amino acid sequences, e.g., because of natural selection processes (*2*). We could not reproduce the high level (>90%) of amino acid sequence identity between the capsid protein (CP) of avian HEV (GenBank accession no. AY535004) and the unpublished GenBank entry swGX32 (accession no. EU366959) claimed by Zhang et al (*3*).

The low level of amino acid sequence identity between rat and human HEV strains might explain the lack of reactivity of transudates from 6 investigated rats in the genotype 1–based ELISA. Consistent with this assumption, a rat hyperimmune serum specimen, raised against a truncated recombinant rat HEV CP derivative, reacted strongly with the homologous antigen but weakly with genotype 3 HEV antigen (P. Dremsek and R.G. Ulrich, unpub. data). Nevertheless, conserved and cross-reactive epitopes have been identified in the CP of HEV (*4*) and can be expected in the antigenic protruding domain of rat HEV CP (*5*). Therefore, some cross-reacting antibodies might exist that would explain detection of rat HEV by the human anti-HEV serum used in immunohistochemical staining.

For sequencing, novel primers were designed (Table). The recombinant nature of strain DQ450072 was not known at time of analysis. Nevertheless, that this sequence clusters near, but not within, the genotype 4 branch is consistent with the reported recombination event.

Virus taxonomy has to "categorize the multitude of known viruses into a single classification scheme that reflects their evolutionary relationships" (*6*). Because the evolutionary relationships could not be determined without sequence analyses, we could not follow the suggestion of Zhang et al. to use other than genetic information for genotype classification (*3*). Future classification of HEV strains would profit from definition of solid criteria and distinct thresholds for definition of genotypes.

Table. Primers used in study (*1*) of amplification of complete genome sequence of rat HEV from rat sample 63, Germany*

Designation	Binding position	Sequence† (5′ → 3′)
rHEV-RACEn‡	307–284	GTGCTCATTAATAGATCGAGGGTG
rHEV-RACE‡	336–313	GGAAGAAAACATCTGTGAATGACA
HEV-100s	78–102	CGGCCAATTCCGCCYTGGSRAATGC
rHEV-900as	905–881	TATGCCCGCCCGCACTAAACAAACT
HEV-800s	776–801	GTGCGGGCCATTGGCTGYCAYTTTGT
rHEV3300as	3099–3074	AGCCGCCATTCTGTTGGCTCCAGATT
rat5-s	2921–2943	CGCCGGGTTGTGATKGAYGAGGC
rHEV-td1-as	4062–4037	GAAATGCCCTGCCCGACCTTGCCATG
HEV-cs	3977–3999	TCGCGCATCACMTTYTTCCARAA
HEV-cas	4446–4424	GCCATGTTCCAGACDGTRTTCCA
Rat HEV-inv-s	4301–4322	GGGGCRCCYGAGTGGATGTGGA
63-5400as	5449–5428	CTCAGTCGCCATGATATGCGTA
#8-ORF2-s	5399–5421	CCCTTACTGCCTYTKCAGGAYGG
#8-ORF2-as	5604–5582	GTGGAAGTGATGGAATTCATRTC
63-5500s§	5555–5576	CAATCCACAACAGTCCCCACGT

*HEV, hepatitis E virus; RACE, rapid amplification of cDNA ends; ORF, open reading frame.
†D = A + G + T; K = G + T ; M = A + C; R = A + G; S = G + C; Y = C + T.
‡Primers were used for 5′-RACE.
§Primer was used for 3′-RACE.

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Antibiotic Resistance: Understanding and Responding to an Emerging Crisis

Karl Drlica and David S. Perlin

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Interest in the problem of antimicrobial drug resistance was once limited to health care workers and academics. Today, the problem is widespread, and patients are acquiring drug-resistant infections for which there may be no active therapeutic agents. Antimicrobial drug–resistant infections, such as methicillin-resistant *Staphylococcus aureus*, have spread beyond health care settings and now cause disease, including life-threatening infections, in community settings.

The goal of Antibiotic Resistance: Understanding and Responding to an Emerging Crisis by Drlica and Perlin is to explain "how human activities contribute to the problem of resistance." The book is intended for "farmers, hospital administrators, government regulators, health department personnel, pharmaceutical executives, and especially individual users" of antibiotics. The book is written in language that readers with a cursory understanding of biology will understand. Readers with little to no biology background can turn to helpful appendixes in the back of the book, where they will find a concise and clear introduction to the Molecules of Life and Microbial Life Forms, which provide the necessary information to tackle antibiotic resistance concepts.

The subject matter was not short-changed in the authors' effort to make it more widely accessible. This book addresses resistance problems in all types of infections: bacterial, viral, parasitic, and fungal. Chapters address diverse issues, including antibiotic drug activity, development, and use; resistance mechanisms and transmission; laboratory detection of resistance; and what we can do to avoid drug-resistant pathogens.

The book's readability is enhanced by the use of text boxes, which provide additional information such as historical antidotes that give depth to the chapter's topic. For example, in Chapter 3, A Survey of Antibiotics, Box 3-2 recounts how a toxic formulation of sulfa drugs in the late 1937, which killed more than 100 persons, led Congress to pass the Food Drug and Cosmetic Act and to the subsequent creation of the Food and Drug Administration.

In addition, each chapter ends with a Perspective section that concisely positions the chapter's topic in the larger world of humans, animals, pathogens, and drugs. This book fulfills its intended purpose and will serve as an important resource for anyone looking for greater understanding of antibiotics and the problem of drug resistance.

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Rembrandt van Rijn (1606–1669). *Aristotle with a Bust of Homer* (1653) Oil on canvas (143.5 cm × 136.5 cm). The Metropolitan Museum of Art, New York, NY

Much have I travel'd in the realms of gold[1]

Polyxeni Potter

"Know thyself" resonated with Rembrandt van Rijn. More than any other artist of his caliber at any time, he explored his own image in as many as 90 self-portraits, some 60 of them paintings, an extraordinary record of self-examination. He was so frank with his depictions he could not have been motivated by narcissism. He may have taken up portraiture for its connection to history painting, a lifelong interest. Portraits were very popular in the commercial market of his day, and his were much sought after in Holland. Whatever the motivation, his self-portraits captured much more than physical features. From youth to ripe old age they amounted to a spiritual autobiography, and since he never strayed more than a few miles from his native Leiden, the journey of discovery was an inward one.

Rembrandt's life has been shrouded in mystery, largely because no written records exist beyond the usual certificates of birth, baptism, marriage, and death. He left no journal, and seven surviving letters from his hand concern routine transactions. But for an inventory of his possessions when near the end of his life he declared himself insolvent, the great artist left few clues about himself, save in his art, a brilliant legacy of more than 2,300 works, among them the famed *Anatomy Lesson of Dr. Tulp*, *Night Watch*, some of his self-portraits, and *Aristotle with a Bust of Homer*, on this month's cover.

Aristotle with a Bust of Homer was commissioned by Don Antonio Ruffo, Sicilian nobleman, art collector, and patron of Rembrandt, without specific guidance, except to paint a philosopher. The meeting of the minds ensemble that ensued was the painter's idea. A mixture of history and myth, the composition contains Rembrandt hallmarks: simplicity, quiet, character, empathy. While two figures are clearly present, a third one, Alexander the Great, appears indirectly, on the ornate pendant worn by Aristotle.

The philosopher is portrayed in his study as a distinguished figure, clad in finery reminiscent of the Renaissance. A certain social rank, the markings of which appear in other works by Rembrandt, including some self-

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[1]John Keats, "On First looking into Chapman's Homer," www.poetryfoundation.org/poem/173746.

portraits, is present in the elegant attire and the sensitive ringed hands. Though not a military man, Aristotle seems decorated, the gold chain and medallion bestowed upon him by the warrior prince displayed prominently. Secure in his own stature, Aristotle seems lost in thought. He rests one hand on the bust as he casts brooding eyes on antiquity's celebrated poet, "deep-brow'd" Homer, a figure much admired by Aristotle and revered by Alexander, Aristotle's pupil, who carried everywhere he went a copy of The Iliad, annotated by his tutor.

This imaginary meeting of three ancient historical figures, a gathering of genius, shows not only the artist's inventiveness and technical brilliance but also his thoughts on the subject. Asked to paint a generic philosopher, he was intrigued by his own choice, and "Like some watcher of the skies/when a new planet swims into his ken," he did much more.

Homer is a legend. The exact period of his life has been debated, his very existence questioned. Skeptics have been so doubtful about him, it has been said in jest that the epic works were not created by him but by someone else with the same name. Still, Homer persists as poet of The Iliad and The Odyssey. Thought to have lived close to 3,000 years ago, he predated realistic portraiture. His image was invented much later and frequently copied, always sightless and bearded, often wearing a headband. Rembrandt likely relied on Hellenistic busts in his own collection for guidance.

The dark and stillness of the room and faint outline of books in back amplify the lighted face and figure of the philosopher. His depiction as a Renaissance man, be it artistic license or intentional anachronism, could not have been more apt. Aristotle knew and understood all that was known in his day, to which he contributed in spades. A man for whom no discipline was uninteresting or unattainable, he was as comfortable with the arts as he was with the sciences.

This extraordinary empirical man paused with humility in front of the revered poet, who explored the mysteries of the human heart. Homer's world, a place of conflict and adversity against which humans were expected to show strength, courage, and perseverance paved the way for philosophy. His was too a world full of wonder and discovery: close calls, shipwrecks, natural catastrophes, lotus-eaters, Cyclops, Sirens, the bravest men, the most beautiful woman, the most faithful wife.

In Rembrandt's portrait, the haunting eyes that surveyed the totality of human knowledge are unfathomable. Contemplation, the philosopher wrote in the Nicomachean Ethics, is the highest form of happiness, and of all pleasures in life, it is the most enduring and self-sufficient. Since the intellect (*νους*) is our most exalted attribute and what it grasps is the highest knowledge, contemplation must be the ultimate form of human activity. Equal to philosophical wisdom, it involves scientific understanding—the intuitive grasp of eternal first principles combined with demonstration.

Contemplation as guide to life has been interpreted in many ways. In the thousands of years since Homer, Aristotle, and Alexander, many have taken the philosopher's call, and some have written modern Odysseys. More than 70,000 species of fungi alone have been described since Aristotle classified living things into animals and plants. His theory of spontaneous generation has been hotly debated. But his concept of emergence holds true: "The whole is not just the sum of its parts" because the emergent order will not arise if the parts simply coexist without interaction.

Complex interactions that Aristotle could not have anticipated, such as cell and solid organ transplantation and antimicrobial drug resistance, continue to stir up our biologic world. In this issue of Emerging Infectious Diseases, incidence of non-*Aspergillus* mold infections in hematopoietic cell and solid organ transplant recipients is increasing, and multiazole resistance in *Aspergillus fumigatus* associated with poor outcome in patients with invasive aspergillosis is now widespread in the Netherlands.

Long after Rembrandt painted Aristotle contemplating Homer, Nikos Kazantzakis wrote his version of The Odyssey. Having called modern humans to action against adversity and even against the inevitability of death, he engaged Homeric language to lead them in Aristotelian contemplation, not for any immediate resolution of perils in the world but as an end in itself: "I know not if I shall ever anchor." Now the day's work is done, "I collect my tools; sight, smell, touch, taste, hearing, intellect. Night has fallen …. I return like a mole to my home, the ground. Not because I am tired and cannot work. I am not tired. But the sun has set."

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EMERGING INFECTIOUS DISEASES

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Upcoming Infectious Disease Activities

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http://www.globalbacteria.org/home

October 12–15, 2011
The Denver TB Course
Denver, CO, USA
http://www.njhealth.org/TBCourse

October 20–23, 2011
49th Annual Meeting of the Infectious Diseases Society of America
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November 6–8, 2011
2011 European Scientific Conference on Applied infectious Diseases Epidemiology (ESCAIDE)
Stockholm, Sweden
http://www.escaide.eu, or email escaide.conference@ecdc.europa.eu

November 16–19, 2011
7th World Congress of the World Society for Pediatric Infectious Diseases (WSPID 2011)
Melbourne, Victoria, Australia
http://www.kenes.com/wspid2011/mailshot/ms3.htm

2012
March 5–8, 2012
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Article Title

Clinical Implications of Azole Resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009

CME Questions

1. Which of the following statements about the prevalence of itraconazole resistance in clinical *Aspergillus fumigatus* isolates is most likely correct?

A. Overall prevalence was 5.3%
B. Resistant isolates were found in half of participating university medical centers
C. The range of prevalence at participating university medical centers was 0%–6%
D. One quarter of azole-resistant isolates had the TR/L98H mutation in the Cyp51A gene

2. A 34-year-old white woman presents with non-Hodgkin lymphoma, pulmonary symptoms, and positive sputum culture for *A. fumigatus*. On the basis of the study by van der Linden and colleagues, which of the following statements about risk factors for development of itraconazole resistance in *A. fumigatus* isolates is most likely correct?

A. History of a hematologic/oncologic disease is not a risk factor
B. Previous azole treatment is necessary for development of resistance
C. Most TR/L98H mutations are resistant to voriconazole and have a narrow range of minimum inhibitory concentrations
D. Azole resistance may develop in patients with cavitary lung lesions such as aspergilloma

3. Which of the following statements about outcomes associated with development of itraconazole resistance in *A. fumigatus* isolates is most likely to apply to the previously described patient?

A. The mortality rate of patients with azole-resistant invasive aspergillosis is ≈50%
B. Two thirds of patients whose treatment was switched to another class of antifungal survived
C. None of the patients who were treated with voriconazole monotherapy were alive at 3 months
D. This study proved that azole resistance causes poor clinical outcomes in patients with azole-resistant aspergillosis

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Invasive Non-*Aspergillus* Mold Infections in Transplant Recipients, United States, 2001–2006

CME Questions

1. You are preparing a 35-year-old woman for hematopoietic cell transplantation (HCT) from a related donor, and you are concerned with the possibility of a fungal infection following transplantation. Which of the following infections was most common among the current cohort of transplant recipients?

A. Scedosporiosis
B. Mucormycosis
C. Chromoblastomycosis
D. Fusariosis

2. Which of the following statements regarding patient characteristics of individuals with fungal infection in the current study is most accurate?

A. All cases had proven invasive fungal infection
B. The overall 90-day mortality rate exceeded 50%
C. Nearly all patients had neutropenia before fungal infection
D. The prevalence of mucormycosis was highest among recipients of HCT from allogenic human leukocyte antigen mismatched related donors

3. What else should you consider regarding factors associated with fungal infection in the current study?

A. Most mold infections occurred in the gastrointestinal tract
B. Liver transplant was particularly associated with a higher risk for mucormycosis
C. There were no cases of late-onset infection
D. Voriconazole failed to prevent many infections, especially mucormycosis

4. Which of the following statements regarding mucormycosis in the current study is most accurate?

A. Rhizopus was the most common genus
B. Renal transplants were associated with the highest incidence of mucormycosis
C. The annual incidence rates of mucormycosis remained stable during the study period
D. Mucormycosis was most common in the months from January to April

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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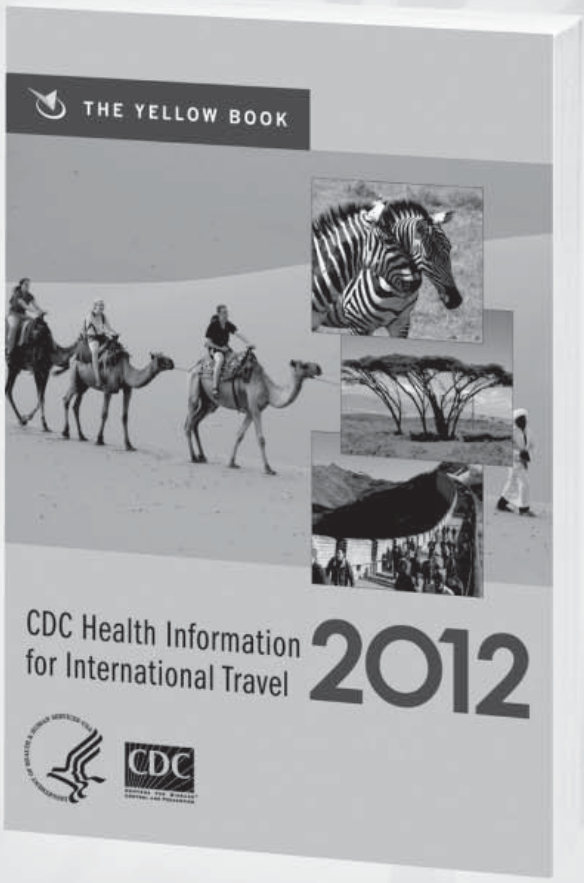

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EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

September 2011

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Bequest of John Ringling, 1936, The John and Mabie Ringling Museum of Art, the State Art Museum of Florida, a division of Florida State University

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

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Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

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Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Figures. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only and should contain 500–1,000 words. They should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.