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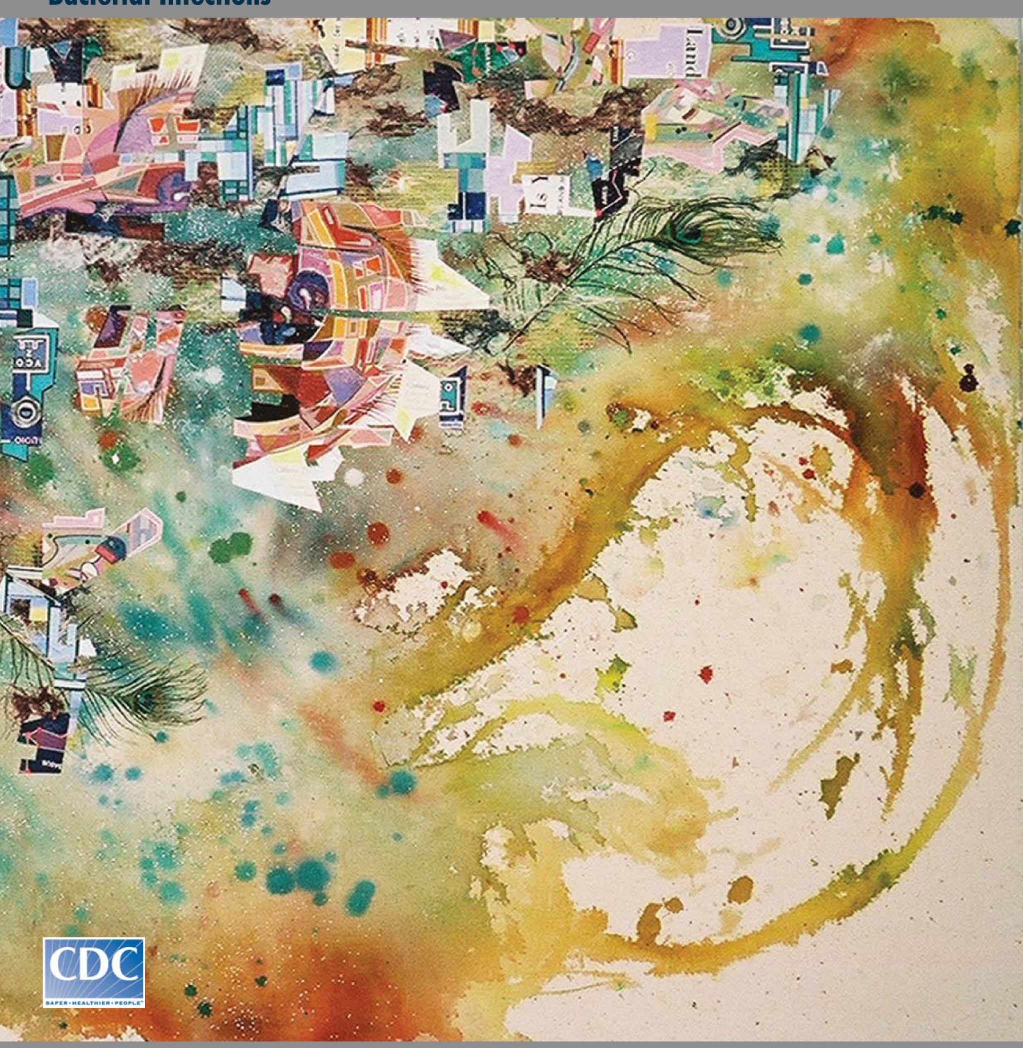

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October 2008

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About the Cover p. 1680

Perspective

Rise and Persistence of Global M1T1 Clone of *Streptococcus pyogenes*....................... 1511
R.K. Aziz and M. Kotb
M1T1 strain, its diversification by phage acquisition, and the in vivo selection of more fit members of its community present an intriguing example of the emergence of hypervirulent forms of a human pathogen.

Research

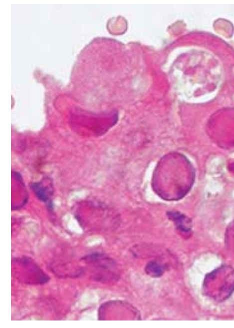
Pandemic Influenza and Excess Intensive-Care Workload 1518
R.E. Nap et al.
Even during the peak of a pandemic, all patients requiring hospital and ICU admission can be served, including those who have non–influenza-related conditions.

Risk Factors for Nipah Virus Encephalitis, Bangladesh 1526
J.M. Montgomery et al.
Patients in Goalando were likely infected by direct contact with fruit bats or their secretions, rather than through contact with an intermediate host.

Deforestation and Vectorial Capacity of *Anopheles gambiae* Giles Mosquitoes in Malaria Transmission, Kenya............................. 1533
Y.A. Afrane et al.
Land use changes are affecting malaria transmission in this region.

p. 1527

p. 1617



Ecological Factors Associated with West Nile Virus Transmission, Northeastern United Sates 1539
H.E. Brown et al.
Risk for disease was 4× greater in the least forested counties.

Deaths from Norovirus among the Elderly, England and Wales............. 1546
J.P. Harris et al.
Norovirus may be associated with ≈80 deaths/year.

Norwalk Virus Shedding after Experimental Human Infection.............. 1553
R.L. Atmar et al.
Noroviruses are shed in feces up to 8 weeks after infection.

Prophylaxis after Exposure to *Coxiella burnetii* 1558
C.E. Moodie et al.
Postexposure prophylaxis may avert Q fever illness and death when the probability of exposure is above the population-specific threshold point.

***Cryptosporidium* Species and Subtypes and Clinical Manifestations in Children, Peru........... 1567**
V.A. Cama et al.
Different genotypes and subtypes are linked to different clinical manifestations.

Epidemic and Endemic Lineages of *Escherichia coli* that Cause Urinary Tract Infections.............. 1575
A.R. Manges et al.
These organisms caused community-acquired infections in unrelated women in 2 distant locations and times.

CME ACTIVITY
Microbial Interactions during Upper Respiratory Tract Infections....... 1584
M.M. Pettigrew et al.
Competitive interactions between bacteria differ by number and species present; thus, vaccination and treatment strategies may alter nasopharyngeal flora and disease susceptibility.

EMERGING INFECTIOUS DISEASES
October 2008

Pyogenic Liver Abscess as Endemic Disease, Taiwan 1592
F.-C. Tsai et al.
The increasing incidence and microbiologic shift might have changed the presentation of pyogenic liver abscess.

Estimating Community *Salmonella*, *Campylobacter*, and Shiga Toxin–producing *Escherichia coli* Infections, Australia 1601
G. Hall et al.
Multipliers that linked surveillance of foodborne diseases with community incidence showed a high prevalence of these diseases.

Dispatches

1610 **Automatic Outbreak Detection Algorithm versus Electronic Reporting System**
M. Straetemans et al.

1613 ***Rickettsia typhi* and *R. felis* in Rat Fleas (*Xenopsylla cheopis*), Oahu, Hawaii**
M.E. Eremeeva et al.

1616 **New Hosts for Equine Herpesvirus 9**
M.D. Schrenzel et al.

1620 **Extended Sequence Typing of *Campylobacter* spp., United Kingdom**
K.E. Dingle et al.

1623 **Chikungunya Outbreak, South India, 2006**
P. Kaur et al.

1626 **Control Strategy for *Echinococcus multilocularis***
D. Hegglin and P. Deplazes

1629 **Mapping the Probability of Schistosomiasis, West Africa**
A.C.A. Clements et al.

1633 **Spinach-associated *Escherichia coli* O157:H7 Outbreak, Utah and New Mexico, 2006**
J. Grant et al.

1637 **Molecular Surveillance for Multidrug-Resistant *Plasmodium falciparum*, Cambodia**
N.K. Shah et al.

1641 **Decreased Tuberculosis Incidence and Declining Clustered Case Rates, Madrid**
J. Iñigo et al.

1644 **Influenza A Virus Infections in Land Birds, People's Republic of China**
A.T. Peterson et al.

1647 **Invasive Group B Streptococcal Infections in Infants, France**
C. Poyart et al.

p. 1625

p. 1627

1650 **Owner Valuation of Rabies Vaccination of Dogs, Chad**
S. Dürr et al.

1653 **Unexplained Deaths and Critical Illnesses of Suspected Infectious Cause, Taiwan, 2000–2005**
T.-H. Wang et al.

1656 **Murine Typhus and Febrile Illness, Nepal**
M.D. Zimmerman et al.

1660 **Effects of School Closures, 2008 Influenza Season, Hong Kong**
B.J. Cowling et al.

Letters

1663 **Age Pattern of Dengue Hemorrhagic Fever, Brazil**

1664 **Confirmed *Mycoplasma pneumoniae* Endocarditis**

1665 **Human Rhinovirus Group C in Children with Lower Respiratory Tract Infection**

1667 **Serogroup A *Neisseria meningitidis* and Reduced Ciprofloxacin Susceptibility**

1669 **Identification of All Dengue Serotypes in Nepal**

1671 **Avian Influenza Virus (H5N1) in Domestic Duck Feathers**

1672 ***Neisseria gonorrhoeae* Meningitis in Pregnant Adolescent**

1674 **Echinococcoses and Tibetan Communities**

1676 **Resource Allocation during an Influenza Pandemic (response)**

Book Reviews

1678 **Risky Trade: Infectious Disease in the Era of Global Trade**

1679 **The Making of a Tropical Disease: A Short History of Malaria**

News & Notes

About the Cover
1680 **Collage and Assemblage in the Microbial World**

Etymologia
1566 ***Coxiella burnetii***

Rise and Persistence of Global M1T1 Clone of *Streptococcus pyogenes*

Ramy K. Aziz and Malak Kotb

The resurgence of severe invasive group A streptococcal infections in the 1980s is a typical example of the reemergence of an infectious disease. We found that this resurgence is a consequence of the diversification of particular strains of the bacteria. Among these strains is a highly virulent subclone of serotype M1T1 that has exhibited unusual epidemiologic features and virulence, unlike all other streptococcal strains. This clonal strain, commonly isolated from both noninvasive and invasive infection cases, is most frequently associated with severe invasive diseases. Because of its unusual prevalence, global spread, and increased virulence, we investigated the unique features that likely confer its unusual properties. In doing so, we found that the increased virulence of this clonal strain can be attributed to its diversification through phage mobilization and its ability to sense and adapt to different host environments; accordingly, the fittest members of this diverse bacterial community are selected to survive and invade host tissue.

Group A streptococci (GAS or *Streptococcus pyogenes*) are strictly human pathogens that normally colonize the throat or skin without causing disease. Members of this species are differentiated into >100 types on the basis of immunogenic differences in their surface M proteins and polymorphisms in the *emm* gene (*1*). The range of GAS diseases is broad and includes both localized and systemic infections that can cause acute or chronic illnesses (Table 1 in online Technical Appendix, available from www.cdc.gov/EID/content/14/10/1511-Techapp.pdf). In most cases, these bacteria cause pharyngitis (sore throat), tonsillitis, or skin infections such as impetigo/pyoderma. At times, however, the bacteria gain access to normally sterile sites and cause invasive disease. Depending on complex host–pathogen interactions, invasive GAS infections can cause either severe shock and multiple organ failure or nonsevere systemic disease, e.g., mild bacteremia and cellulitis (*2*,*3*). Likewise, invasive infections of soft tissues can be severe, e.g., necrotizing fasciitis (NF), or nonsevere, e.g., cellulitis or erysipelas (*4*). Whereas host genetic susceptibility plays a key role in modulating disease manifestation, variations in bacterial virulence properties contribute to infection severity.

Despite reports that particular serotypes or *emm* types are more commonly associated than others with particular disease manifestations, serotypic designation does not always reflect the pathogenic potential of a given strain. As we discuss below, serotype diversification can convert relatively avirulent serotypes to highly virulent ones. Dissection of molecular and genetic events leading to such diversification provides insight into how the changes in pathogenesis and host–pathogen interactions can lead to the resurgence of a severe infectious disease.

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Resurgence of Severe Invasive Streptococcal Diseases and Emergence of Highly Virulent GAS Strains

In the 19th century, GAS infections were associated with severe and frequent epidemics of invasive and often fatal illnesses, including a pandemic of scarlet fever in the United States and Great Britain (*5*). Invasive GAS infections with severe manifestations continued through the 1920s (*5*). The severity of these illnesses then declined notably until the early 1980s, when a statistically significant simultaneous recrudescence of the severe and fatal forms of invasive GAS infections occurred in different parts of the

industrialized world (*6*,*7*). Accordingly, in 1993 a working group developed the case definition for streptococcal toxic-shock syndrome (STSS) as hypotension accompanied by multiple organ failure, indicated by 2 of the following signs: renal impairment, coagulopathy, liver involvement, adult respiratory distress syndrome, a generalized rash, and soft tissue necrosis (*8*). A modified definition of STSS was later adopted to focus on the host immune-mediated severe systemic disease associated with invasive infections, manifested by hypotension and multiple organ failure, excluding skin rash, soft-tissue necrosis, and gangrene (*2*). Similarly, NF was defined by the histopathologic identification of necrosis of superficial fascia and a polymorphonuclear infiltrate and edema of the reticular dermis, subcutaneous fat, and superficial fascia (*9*,*10*). The speed and rigor by which invasive GAS infections spread in the host, sometimes causing severe damage to the fascia and muscles, prompted its designation as the "flesh-eating disease."

Epidemiologic studies showed that the resurgence of severe invasive GAS infection was not limited to sporadic cases; rather, it represented a global spread, ushering in a new pandemic, similar to that reported in the earlier part of the 20th century. An important feature of this latest pandemic is its association with a distinct epidemiologic shift in GAS serotypes. Although many GAS serotypes are capable of causing severe diseases, a few were more frequently isolated from patients with severe cases, e.g., M1, M3, M18, and M28 strains (Table 2 in online Technical Appendix). However, whether those serotypes cause more severe disease because of their hypervirulence or because they were also the most prevalently isolated strains in the community at that time was not clear (*11*,*12*). These possibilities are not mutually exclusive, but in fact may be related. We believe that the unique features of the newly emerged subclones of GAS serotypes, in particular the M1T1 clonal strain, evolved as a result of diversification of the bacteria and acquisition of new genes that improved their fitness to infect humans. This, together with host-imposed pressure, resulted in the selection of hypervirulent mutants of this strain associated with an ability to cause severe forms of the invasive infection in susceptible persons.

Features of the Newly Emerged Hypervirulent Global M1T1 Strain

Whereas most GAS serotypes traditionally exhibit cyclic epidemiologic patterns, appearing and disappearing from the community at different times (*13*), the M1T1 subclone has persisted globally for more than a quarter of a century as the most frequently isolated serotype from patients with invasive and noninvasive cases. Advanced molecular and genomic tools showed a great deal of diversity among GAS strains belonging to the same serotype, and the M1 serotype is no exception. The clonality of the reemerged M1T1 strain was first described by Cleary et al. (*14*) and later confirmed by others by the use of different molecular methods (Table 3 in online Technical Appendix), which confirmed that the M1T1 clone differs from its ancestral M1 clone in several aspects. We will present evidence that those differences have indeed contributed to the stark difference in epidemiologic and virulence properties between 2 strains belonging to the same serotype.

Together with the Ontario Streptococcal Study Group and the Centers for Disease Control and Prevention, our laboratory launched one of the earliest and most comprehensive prospective studies of invasive GAS pathogenesis in Ontario (*8*), where active surveillance of invasive GAS cases took place during 1992–2002 (*4*,*9*). M1T1 isolates recovered from patients with noninvasive as well as invasive cases, of varying severity, were extensively analyzed at the molecular level and shown to be clonal regardless of case severity (*3*). This clonal M1T1 strain possesses the *emm1.0* allele of the M1 gene (*3*) and is one of the opacity factor–negative GAS serotypes. This strain differs in its virulence and genomic content from other less virulent M1 strains, represented by strain M1 SF370, the first fully sequenced GAS strain (*15*).

Several events appear to have contributed to the diversification of the M1 GAS serotype, leading to the emergence of the M1T1 global strain. Specifically, diversification through the loss and/or acquisition of phages that took away certain genes and introduced new ones into the M1 serotype is a major contributor to the emergence of this strain. This phenomenon is certainly not unique to the M1T1 strain, but is also seen in the M3T3 and M18 strains (*16*,*17*), which co-emerged with the M1T1 clonal strain in the 1980s.

Contribution of Prophages to Emergence of Global M1T1 Strain

In 1996, Cleary et al. found that the globally disseminated M1T1 differs from its closely related M1 subtypes by 70 kb of phage DNA (*18*). Ensuing studies from our group, in which we conducted global genomic comparison of the M1T1 clones and the closely related M1 SF370 strain, demonstrated that most of the genetic differences (≈5% divergence) were accounted for by phage or phage-like sequences. After assembling these distinct sequences, we identified 2 novel prophages that were introduced into the M1T1 global strain (*19*). One prophage (SPhinX) carries the *speA2* gene, which encodes the potent superantigen SpeA; the other (PhiRamid) carries the *sda1* gene, which encodes the most potent streptococcal nuclease identified thus far (*19*,*20*). The introduction of these phages into the M1T1 clonal strain was later confirmed by the complete genome sequence of a clinical M1T1 isolate, MGAS5005 (*21*).

The M1T1 prophages exhibit considerable genetic mosaicism, and the sequence analysis of the 2 novel M1T1 phages demonstrates that these bacterial viruses continuously exchange functional modules by various genetic mechanisms, including different modes of recombination (*19*). We believe that exchange between the lysis and lysogenic conversion modules of GAS prophages has led to the swapping of virulence genes (toxins) among phages (*19*). We also believe that this process is facilitated by a highly conserved gene, paratox (*prx*), commonly found between the toxin gene and phage attachment site. Conserved *prx* sequences on 1 side of the toxin gene together with 1–3 highly conserved phage genes on the other side (lysin, holin, and/or hyaluronidase genes) are likely to facilitate recombination events leading to swapping of toxin genes among bacterial isolates (Figure 1) (*19*). This notion is supported by the fact that strains belonging to the same serotype may have different virulence components carried by the same or highly similar phages, whereas those belonging to different serotypes may, in fact, have identical phage-encoded toxins. For example, 4 highly similar phages (370.3, 5005.2, MemPhiS, 315.3) identified in M1 SF370, M1T1 5005, M1T1 6050, and M3 strains, respectively, have different DNases in their lysogenic conversion modules. Phages 370.3 and 5005.2 are >99% identical to each other and carry the *mf3* gene, and each is 90% identical to MemPhiS and 315.3, which carry the *mf4* gene instead (Figure 2).

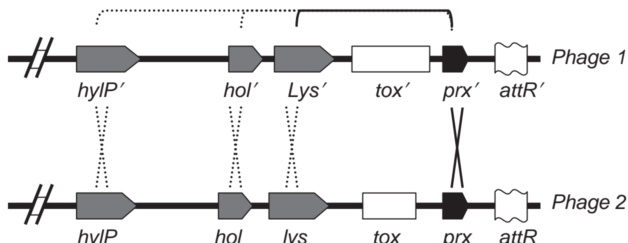

Figure 1. Suggested model for toxin mobilization between phages, reprinted from Aziz et al. (*19*). Recombination hot spots on both sides of the toxin genes are shown: one is *prx* (paratox), and the other may be *lys* (lysin), *hol* (holin), or *hylP* (phage hyaluronidase).

Acquisition of Novel Virulence Genes by Global M1T1 Strain and Effect on Virulence

Two virulence genes, *speA2* and *sda1*, were introduced into the M1T1 strain by prophages and are likely to have contributed to its increased fitness and virulence (*19*,*21*). SpeA2 is an important and potent streptococcal superantigen. Although GAS has a rich superantigen repertoire, different strains harbor different combinations of superantigen genes—some are phage encoded, while others are integrated into the bacterial chromosome. Both the global M1T1 strain and its ancestral SF370 (*15*) strain have the superantigen-encoding genes *speF*, *speG*, *speJ*, and *smeZ1*. However, these 2 strains differ in that the global M1T1 strain has *speA2,* whereas SF370 has *speC* (Table). Both SpeA and SpeC are prophage-encoded, whereas the other M1T1 superantigens are chromosomal. Additionally, the clonal M1T1 strain lacks *speH* and *speI*, which are encoded on a single phage in M1 SF370 (*15*,*21*).

Of particular relevance to this discussion is that the *speA* gene was seen in M1 isolates obtained in the early 20th century but had almost vanished from M1 isolates obtained between the 1920s and early 1980s. The loss of *speA* was thought to be one of the main reasons for the sharp decline in severe invasive GAS infections during this time (*23*,*24*). Likewise, the reintroduction of the *speA2* allele in the M1T1 clonal strain in the 1980s prompted speculations that SpeA, and in particular its allelic variant SpeA2, was a major factor in the resurgence of severe invasive GAS infections during that time (*25*). However, additional studies showed that, although the reintroduction of *speA* may have been a factor, the acquisition of other virulence genes by the M1T1 clone is more likely to have had a more profound effect on its increased fitness and virulence in vivo (*20*,*26*,*27*). Nonetheless, the fact that SpeA was missing from most GAS isolates for >50 years suggests that the reintroduction of this superantigen may have increased the risk for persons to have invasive infections because they

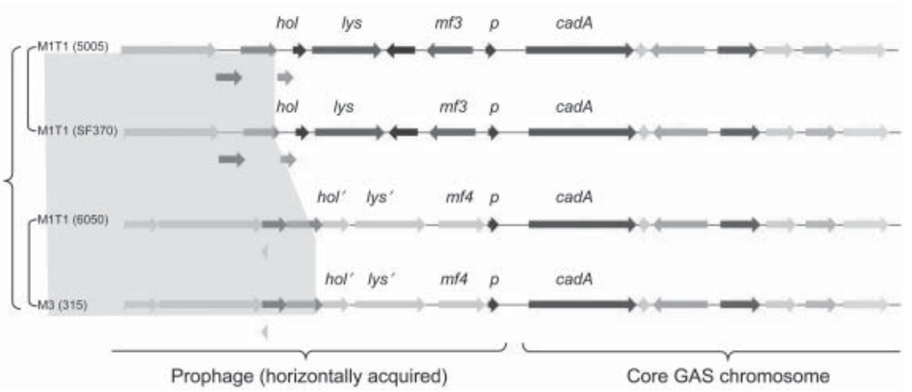

Figure 2. Similarities and differences between the 4 highly related prophages 5005.2, 370.3, MemPhiS, and 315.3. The figure, generated by the SEED comparison tools (*22*) (http://theseed.uchicago.edu), shows the physical maps of the 4 prophages near their attachment sites. Arrows with identical colors designate orthologous genes; those in gray designate alternative alleles of the genes. *p*, *prx; mf*, mitogenic factor; *cadA*, heavy metal/cadmium transporter ATPase; GAS, group A streptococci.

Table. Genomic differences between M1T1 and M1 SF370*

Difference	M1T1	SF370
Prophages or prophage remnants	SPhinX (*speA*2), MemPhiS (*mf3/mf4*), PhiRamid (*sda1*)	370.1 (*mf2, speC*), 370.2 (*speH, speI*), 370.3 (*mf3*), 370.4 (phage remnant)
Superantigen genes	***speA2***, *speG*, *speJ*, *smeZ1,*	***speC***, *speG*, ***speH***, ***speI***, *speJ*, *smeZ1*
Streptodornases	*mf/spd*, *mf3/spd OR* ***mf4/spd4***, ***sda1***	*mf/spd*, ***mf2***, *mf3/spd3*
Other	Insertion sequence (IS1548), SNP in the SLO/NADGH region	

*SNP, single nucleotide polymorphism; SLO, streptolysin O; NADGH, nicotinamide glycohydrolase. Toxin gene names in **boldface** designate genes that are unique to either strain.

lack antibodies that neutralize its superantigenic activity. Indeed, the lack of superantigen-neutralizing antibodies has been shown to increase the risk for invasive disease (*28*,*29*).

Sda1, which was also acquired by the M1T1 global strain, is a potent streptodornase (streptococcal nuclease) (*20*) and is not found in most of the other prevalent strains but has been recently reported in an M12 strain (*16*). Streptodornases are secreted extracellular nucleases classically thought to play an important role in virulence by degrading pus (*30*). Every GAS serotype sequenced so far contains ≥1 streptodornase paralog. These various streptodornases, which differ in the pH optima for their nuclease activity, are likely functionally nonredundant, possibly having different substrate specificity, and may be differentially active in certain host niches or at different times during the infection. The M1T1 clone has, in addition to *sda1*, the chromosomal streptodornase *spd/mf* (alias *speF*) and another phage-encoded streptodornase, *spd3*/*mf3* (or—less frequently—*spd4/mf4*); however, it lacks *spd2*/*mf2* found in the M1 SF370 (*19*,*20*). Despite the presence of multiple DNases in the bacteria, Sda1 has the highest specific activity among the streptococcal nucleases. We showed that the increased activity of Sda1 has resulted from a frame-shift mutation in its C terminus, and when the additional C-terminal sequence of Sdal was deleted, the enzyme activity dropped significantly (*20*).

Sda1, unlike the other nucleases, appears to play a major role in virulence, and inactivating its gene resulted in a dramatic loss of virulence (*26*,*27*,*31*), whereas introducing it into an avirulent strain led to a virulent phenotype (*26*). Sdal protects bacteria against neutrophils (*31*)—which entrap the bacteria in neutrophil extracellular traps (NETs) (*32*)—by degrading these DNA NETs, thereby freeing the bacteria and promoting their ability to invade host tissues (*26*). Additionally, recent evidence suggests that the Sda1 expression may synergize with host factors, leading to additional selective pressure on the bacteria in vivo and resulting in the emergence of a hypervirulent phenotype of the same bacteria (*27*).

Besides the exchange of phage-encoded toxins, additional recombination events may have contributed to the diversification of the M1T1 clone. In a recent study, Sumby et al. (*21*) used DNA–DNA hybridization and single nucleotide polymorphism analysis to show that a 36-kb chromosomal region has been horizontally transferred to M1T1 by recombinatorial replacement from an M12 ancestral strain. This chromosomal region harbors genes encoding 2 important toxins, streptolysin O (SLO) and nicotinamide glycohydrolase (NADGH or NADase), both of which were more highly expressed in M1T1-MGAS5005 compared to M1 SF370 (*21*). Although these differences in expression might be a consequence of the recombination event, we believe that the enhanced expression of these genes is more likely due to a mutation in the *covS* gene of the studied MGAS5005 strain, which resulted in higher expression of virulence networks (*33*). SLO is an important GAS cytolysin that enhances cytotoxicity and toxin translocation (*34*,*35*), and its heightened expression would be expected to increase virulence. It is therefore apparent that several mechanisms led to GAS diversification and that the globally disseminated M1T1 clone has acquired several virulence factors that seem to have contributed to its unusual persistence, spread, and virulence.

In vivo Selection of Hypervirulent Descendents of Global M1T1 Strain

In addition to the introduction and loss of specific genes in the global M1T1 strain, a high degree of variability in the expression of virulence genes among isolates belonging to this clonal strain was reported (*3*,*36*). This variable expression, in part, depended on where and when the isolates were recovered from the host. However, one of the most notable changes in gene expression that arises in response to host environmental pressure is the remarkable downregulation of the major streptococcal protease, SpeB, and the consequent significant increase in bacterial invasion and severity of GAS sepsis (*27*,*37*,*38*).

Earlier studies by Kansal et al. (*37*) provided the first hint for the reciprocal relation between SpeB expression and severity of GAS sepsis, when they observed that isolates recovered from patients with more severe cases expressed no, or significantly less, SpeB compared to those recovered from patients with nonsevere cases. In ensuing studies, we found that M1T1 regulates its secreted proteins by at least 2 mechanisms (*39*), a transcriptional regulation,

and a posttranslational degradation and remodeling of bacterial proteins by SpeB, that, itself, is tightly regulated (*40*; online Technical Appendix, supplementary reference *41*). The secreted proteome in the presence and absence of active SpeB is starkly different. Essentially most extracellular virulence factors, including M protein, streptokinase, SpeF, Sda1, C5a peptidase, and the secreted inhibitor of complement, are degraded by this protease, resulting in decreased virulence. The advantage of this massive degradation of virulence factors to the bacteria is not entirely known, but we predict that this may be a means by which the bacteria camouflage themselves from the host during the initial stages of infection. By degrading their virulence components, bacteria may evade initial innate host defenses at the site of the infection until they gain access to a host niche (e.g., skin), where they can start to multiply. Thus, SpeB may facilitate the initial invasion of bacteria through its proteolytic action on host matrix proteins. However, within 60–80 hours after infection, the bacteria are subjected to a hostile human environment, and consequently, there is a selection for more fit mutants within the bacterial community that are better adapted to confront host defenses and gain access to blood and possibly other sterile sites. The more fit mutants, it turns out, are those that lack SpeB expression because of a mutation in *covS*, which is a part of a 2-component regulatory system (CovRS) involved in regulating 15% of GAS genes including SpeB (online Technical Appendix, supplementary reference *42*). Indeed, recent studies provided evidence for the co-existence of at least 2 very different phenotypic forms of M1T1 bacteria in the initial stages of infection through the skin of mice characterized by $SpeB^+$ or $SpeB^-$ phenotypes (*27*,*39*).

The downregulation of SpeB spares several key virulence factors that include Sda1 and streptokinase. Our recent studies showed that sparing Sda1 frees the bacteria from neutrophil NETs (*27*). Similarly, in a human plasminogen-transgenic mouse model, sparing streptokinase allowed accumulation of surface plasmin activity and increased bacterial evasion (online Technical Appendix, supplementary reference *43*). Additional differentially expressed genes in the in vivo–selected *covS* mutants are also likely to contribute to increased virulence, and these are currently being investigated.

M1T1 and the Future of GAS Epidemiology

Is there an exit plan for M1T1? How long will this strain survive and prevail? Will there be another prevalent strain in the future? It is intriguing that although M1T1 causes deadly conditions, this clone keeps infecting many persons, retaining its superior prevalence. This suggests that there is an exit plan for this clone, or that it is so widely spread among the human population that it keeps being transmitted through genetically protected persons, who serve as reservoirs for it. The diversity within the bacterial population in the host also suggests that while hypervirulent mutants cause deadly diseases when the bacteria invade unusual niches, the less virulent members of the same population survive well in the primary niche (e.g., the throat or nasopharynx) and thus could drive the disease transmission.

Several potentially interactive factors may have contributed to the persistence of M1T1 and may maintain this strain for a long time. These factors include the acquisition of new virulence genes and the differential regulation and expression of virulence genes caused by selection of mutants within the microbial community. These changes in the pathogen, as well as changes in herd immunity and differential host susceptibility, are likely to create dynamic interactions between streptococci and their human host.

When novel strains or clones emerge that express novel proteins or variants of old proteins, these strains are endowed with the ability to better withstand the pressure of herd immunity. According to this hypothesis, M1T1 and other strains that reemerged in the mid-1980s may have successfully survived herd immunity either because they acquired new protein-encoding genes or because they possessed allelic variants of key genes encoding proteins and/or novel alleles that were as-yet unsampled by the immune system. Also, the acquisition of new genes or the sparing of existing proteins from proteolytic degradation may have endowed the bacteria with means to better evade host immune defenses.

In summary, we believe that the emergence of the M1T1 strain, its diversification by phage acquisition, and the in vivo selection of more fit members of its community present an intriguing example of molecular events that can drastically change the epidemiology and virulence of an otherwise avirulent or less virulent organism. Predicting whether other GAS strains may follow a similar trajectory to M1T1 is difficult: The next prevalent strain to emerge may have to combine changes in chromosomal and phage-encoded genes to enhance its fitness and allow it to adapt to different host environments; it also has to be resistant enough to phage-driven lysis. (More prophages enrich the bacteria with additional toxins, but they may also bring the potential risk of lysing the bacteria at any time a phage is induced.) As there are now more and more examples of phage exchange even within and between different bacterial species (online Technical Appendix, supplementary reference *44*), the traditional classification schema may have to be replaced by ones that better reflect the bacterial virulome. This virulome, as discussed here, can be grossly altered, depending on the environment the bacteria face and the consequent selection of underrepresented minority of the bacterial community that is best adapted to deal with various hostile host milieus.

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Pandemic Influenza and Excess Intensive-Care Workload

Raoul E. Nap, Maarten P.H.M. Andriessen, Nico E.L. Meessen, Dinis dos Reis Miranda, and Tjip S. van der Werf

In the Netherlands a major part of preparedness planning for an epidemic or pandemic consists of maintaining essential public services, e.g., by the police, fire departments, army personnel, and healthcare workers. We provide estimates for peak demand for healthcare workers, factoring in healthcare worker absenteeism and using estimates from published epidemiologic models on the expected evolution of pandemic influenza in relation to the impact on peak surge capacity of healthcare facilities and intensive care units (ICUs). Using various published scenarios, we estimate their effect in increasing the availability of healthcare workers for duty during a pandemic. We show that even during the peak of the pandemic, all patients requiring hospital and ICU admission can be served, including those who have non–influenza-related conditions. For this rigorous task differentiation, clear hierarchical management, unambiguous communication, and discipline are essential and we recommend informing and training non-ICU healthcare workers for duties in the ICU.

In the Netherlands a major part of preparedness planning for an epidemic or pandemic, e.g., avian influenza A, consists of maintaining essential services provided by the police, fire departments, army personnel, and healthcare workers (HCWs). Even if an effective vaccine against avian influenza (H5N1) would be available (*1*), preparation for a pandemic is still vital to maintain optimal care for acute-care patients and those with influenza-like illness (ILI). The preparation for excess workloads among HCWs becomes even more important with the emergence of highly pathogenic avian influenza strains.

We present a model to show the impact of the increased demand in HCWs with the increase in the number of hospitalized patients. We factor in the notion that the number of HCWs will be reduced because of increased absenteeism, which in turn affects the utilization of intensive-care beds and mechanical ventilation capacity. We present scenarios aiding in temporarily increasing the work force of HCWs in the intensive-care unit (ICU) environment using different additive strategies. Because the surge capacity of intensive-care resources is typically limited (*2*), we explore what training and preparation HCWs and managers at different levels will need to face the challenges posed by pandemic influenza.

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Methods

Setting

The University Medical Center Groningen (UMCG) is a tertiary-care university hospital covering ≈12% of the total Dutch population and ≈30% of the total surface area of the Netherlands. Under Dutch law, UMCG plays a dominant role in the region to organize and coordinate healthcare surge capacity during a catastrophe such as an avian influenza pandemic. With regional and municipal health authorities, general practitioners, and medical and managerial representatives of all 15 hospitals in the northern Netherlands region, training courses were organized for pandemic influenza. These courses emphasized the need for enhanced collaboration, sharing of information, and communication. Part of this training course was the development of an epidemiologic model to access the regional impact of a pandemic and the extent of possible preparations (*3*) at both managerial and medical domains.

Basic Model

We used FluSurge 2.0 (*4*) and a computer model in an Excel (Microsoft, Redmond, WA, USA) file developed by one of the authors to calculate the impact of an influenza

pandemic in the Netherlands on hospital admission and occupancy rate of all ICU beds (i.e., those with facilities for mechanical ventilation) (*3,5*). Data on population (slightly >1.7 million) and age distribution were obtained from publicly available sources. Because age distribution in the Dutch population data was provided in blocks of 5 years, we converted these data to an even distribution to enable calculations with the FluSurge program (*6*). Data on total hospital beds, ICU beds, and number of nurses and their fulltime equivalents were obtained from publicly available sources (*7*). Information on ICU capacity was also obtained from reports from hospital administrators during the training sessions. These data on reported ICU capacity were discussed during a semistructured telephone interview with intensive-care specialists (usually anesthetists or internists) in August 2006. On the basis of these data, we estimated the regular bed capacity and maximal surge capacity. Numbers on the effects of pandemic influenza on healthcare services were adopted from the National Institute for Public Health and the Environment (RIVM) (*5,8*). RIVM presented tables for 25% and 50% disease attack rates (ARs) that represented best and worst case scenarios. From these tables, we calculated the 30% AR by linear transformation. A 30% AR is the most likely scenario according to the Centers for Disease Control and Prevention and RIVM. The AR was defined as the percentage of the population that became ill.

We also calculated within the model the total number of patients admitted to the hospitals at each point in time during the pandemic. We defined the first day (day 0) as the moment the World Health Organization (WHO) declares effective human-to-human transmission (phase IV or V in the current WHO phase of pandemic alert). We assume the pandemic would reach the Netherlands ≈15 days after this first confirmed, effective human-to-human transmission.

We took into account the time each patient would occupy a hospital or ICU bed (range 8–15 days), based on experience with patients admitted to ICU with diagnoses of pneumonia or sepsis. Finally, we incorporated estimated risk for death per patient, a rate that in turn would reduce the number of admitted patients at any 1 time. Because RIVM data are in weekly blocks, we evenly distributed the number of hospital admissions and deaths during weekdays. We also factored in our calculations the effect of treatment (within 48 hours of infection) with antiviral medication on the spread and impact of the pandemic, although the exact effect size is still uncertain (*6,9*). Antiviral medication is assumed to reduce the total number of hospital admissions by 50% and death by ≈30%.

In the basic model we incorporated the probable absenteeism of HCWs attributable to illness. We assumed that HCWs will become ill at a similar rate as the normal population. We used the total number of HCWs and linearly transformed the ratios of illness and death of the population over time on the HCW database. We focused our preparedness plan on adults and assumed a pattern in the outbreak similar to that for Spanish flu (*10*) and severe acute respiratory syndrome, in which adolescents and adults accounted for most patients.

Modeling Nursing Workload

We developed an extension to this model that showed the effects of increased demand in the HCW force because of an increased number of hospitalized patients with ILI. In this model, we factored in the notion that the number of available key HCWs (e.g., physicians, intensive-care nurses) would be reduced because of ILI absenteeism, which affects the use of ICU beds and mechanical ventilation capacity.

Furthermore, using different additive strategies reported in the literature, we present several alternatives to increase the number of available HCWs. All 3 scenarios were adopted because of their inherent ease of use in daily hospital practice.

We used the basic model (*3,5*) for peak hospital and ICU occupancy rates during a pandemic of influenza. A major part of preparation encompasses clearing all 15 regional hospitals of general surgical and medical patients, leaving ≈30% of acute-care capacity for non–influenza-related illness. In these 15 hospitals, the total ICU capacity is 200 beds. The predominant use of ICU beds in Europe is for postoperative care (instead of recovery rooms or post-anesthesia special-care units) (*11*). Of these, 60 ICU beds (30%) need to be reserved for acute care for non–influenza-related illness, leaving ≈140 ICU beds for influenza-related patients. The mean ICU length of stay for postoperative and general medical care patients is 3.4 (median 1 day, standard deviation [SD] 7.4) days. Therefore, it follows that ≈96% of these 140 ICU beds occupied by postoperative and general medical care patients at the beginning of the pandemic can be made available within a 14-day period for patients ill from flu.

Even though we assume HCWs will become ill at the same rate as the general population, we also assume that morale and professionalism will be high and that undue absenteeism will not occur. That is, adherence to professional standards of HCWs reporting for duty will be high, although some unavailability of HCWs might be expected because of care duties within families or among neighbors who may become ill (*12*). This erosion has not been taken into account in the model we present.

To model the available number of HCWs in relation to the maximum number needed in a pandemic, we translated the peak ICU occupancy rate into a severity of illness and workload scoring system. A variety of validated scoring systems for patient severity and workload exist for use in the ICU environment and are widely used (*13–18*).

We used data derived from the databases of the European Research in Intensive Care Units (EURICUS) projects, a multicenter project designed to study the ICUs of the European Union (EU), to project severity of illness, diagnosis, and workload data in the ICU. A description of the methods, objectives, and results of these projects have been reported (*11,15,19,20*). These projects were developed from 1994 through 2000 and included 137 ICUs from 14 different EU states with a total of 43,027 individual patient records and 227,209 nursing days. Many study variables were analyzed, including age, diagnosis, length of stay, Simplified Acute Physiology Score (SAPS-II) (*14*), Sequential Organ Dysfunction Score (SOFA-II) (*21*), ICU mortality rate, hospital mortality rate, and Glasgow Coma Score. Part of this research was the measurement of nursing workload in ICUs (*15–17*). Nursing workload per intensive-care patient was calculated by using the Nine Equivalents of Nursing Manpower use score (NEMS) (*16*). NEMS is a therapeutic index to measure nursing workload at the ICU level. The use of NEMS has been developed and validated for multicenter ICU studies; management purposes in the general (macro) evaluation and comparison of workload at the ICU level; and prediction of workload and planning of nursing staff allocation at the individual patient level. NEMS correlates highly with all currently used severity of illness scoring systems such as Acute Physiology, Age, Chronic Health Evaluation III, SAPS-II, and SOFA-II (*16,20*). The major advantage of all these scoring systems is that they are an objective and reproducible measure of nursing workload related to the various activities performed in the ICU, without considering the appropriateness of standing policies of medical care.

We attributed scores on the basis of a specific diagnostic category (*13*) to the corresponding NEMS points from the EURICUS projects database. We used the medical diagnostic category “bacterial/viral pneumonia” (diagnosis category 15) as an indicator for nursing care requirements of ILI patients, as this closely matches the medical condition typical for influenza patients. Furthermore, we used the average NEMS points for all patients (minus the patients with diagnosis 15) in the EURICUS database as an indicator for the nursing workload necessary for the non-ILI acute-care patients. We modeled the available HCWs with a 30% AR, 25% and 50% ICU admission rate, and mean length of stay of 8 days and 15 days without antiviral medication (AVM) (pandemic period 9 weeks) and 30% AR, 25% and 50% ICU admission rate, and mean length of stay of 8 days or 15 days with AVM (pandemic period 14 weeks).

Scenarios

Scenario 1

Increasing the number of HCWs by expanding the work shift from 8 to 12 hours would increase the number of operational HCWs by ≈50%. The huge strain on personnel is justified, when one considers the relatively short peak surge period, and we expect HCWs to comply.

Scenario 2

HCWs will become sick with ILI at the same rate as the general population. Lee and Chen (*22*) showed that 8 weeks’ prophylactic use of neuraminidase inhibitors decreased peak absenteeism among HCWs from 10% to 2%. We translated this in our model by reducing the number of lost NEMS points (e.g., number of lost HCWs) by 80% for the entire pandemic period.

Scenario 3

In an international ICU workload study, Miranda et al. (*17*) showed that tasks performed in normal ICU environment by ICU nurses are only 30% dedicated to technical and specific ICU tasks for which ICU nurses are trained. This result means 70% of all tasks in ICU are regular nursing tasks performed daily in non-ICU, standard healthcare hospital environments. In this scenario, we mathematically decreased the number of NEMS points to be covered by ICU nurses to 30%. Non-ICU nurses and other hospital personnel (communication advisors, psychologists, physicians, administrative staff, and the like) have to be prepared and trained to take over, for a relatively short period, the 70% of nontechnical duties regularly performed by ICU nurses. We assume that there will be substantial numbers of HCWs in wards and ancillary facilities available because all hospitals involved will stop admitting postoperative care and non–influenza-related medical patients when WHO declares the pandemic alert. This will free ≈70% of all HCWs in the 15 hospitals.

Scenario 4

Finally, we combined the effects of all 3 scenarios in an overall model of available HCWs. This final model gives the opportunity to study the effect of 3 easy-to-implement scenarios.

Results

The Table shows the result of our baseline calculations. For the 30% non-ILI acute-care patients, we attributed 24.8 (SD 9.9) mean NEMS points; for the ILI patients, we attributed 28.6 (SD 9.8) mean NEMS points. We assume that an ICU nurse can deliver 46.3 NEMS points in 24 hours. Because HCWs will become ill as the population does, the number of NEMS points available will be reduced as the pandemic period progresses; the lowest number will occur at 28 days, when the pandemic period is assumed to be at its highest point (online Appendix Table 1, available from www.cdc.gov/EID/content/14/10/1518-appT1.htm). The lowest number of NEMS points will occur at day 43, when AVM

Table. Study population characteristics from EURICUS projects*

Characteristic	Study population	Diagnosis category 15†
Total no.	39,158	1,413
ICU length of stay, mean (SD)	5.2 (10.4)	10.5 (13)
Age, no. (%)	59.3 (19.8)	57.7 (21.8)
SAPS-II, no. (%)	31.9 (17.8)	39.5 (18.8)
NEMS, no. (%)	24.8 (9.9)	28.6 (9.8)
ICU deaths, %	13	27
Overall deaths, %	19	36

*ICU, intensive care unit; SAPS, Simplified Acute Physiology Score; NEMS, Nine Equivalents of Nursing Manpower use score.
†Values are given as mean (SD).

is available for the population (online Appendix Table 2, available from www.cdc.gov/EID/content/14/10/1518-appT2.htm). As can be seen from both online Appendix Tables, HCW shortage will occur if the ICU admission rate increases to 50% (mean length of stay 8 days or 15 days without AVM). However, if AVM is available, no HCW shortage will occur, under the premises that all general medicine and surgery have been temporarily stopped.

Scenarios

Scenario 1

In Figure 1, panels A and B, the results are given for the model length of stay of 15 days, with 25% and 50% ICU admission rates with and without AVM. Even in the worst case scenario, a 15-day length of stay without availability of AVM for the individual patient (Figure 1, panel A), no staff shortage appears. The models for a length of stay of 8 days, 25% and 50% ICU admission with and without AVM (not shown), show no staff shortage over time.

Scenario 2

If the number of HCWs who become ill from ILI is reduced by 80%, a similar increase of deliverable NEMS points is achieved. This increase is irrespective of the number of patients admitted to the hospital and ICU. In Figure 2, panels A and B, results are given for the models with a 15-day length of stay, 25% and 50% ICU admission rate, with and without AVM. In this model, the effect of potential HCW staff shortage is most profound at a 50% ICU admission rate. Even decreasing the number of ill HCWs because of 8 weeks' prophylactic use of neuraminidase inhibitors (*22*) does not solve staff shortage when it is most needed.

Scenario 3

Transfer of tasks to other HCWs than ICU nurses decreases the number of NEMS points needed by 70%. We assume acute-care and ILI patients admitted to the ICU will require the 30% technical ICU-related work of ICU nurses. This decrease in the number of NEMS points needed has a direct effect on the necessary workload in the different AR and length-of-stay models. Figure 3, panels A and B, shows that decreasing the number of required NEMS in the ICU results in sufficient numbers of HCWs being available for care of ILI patients.

Scenario 4

We combined all 3 scenarios to access the impact of the 3 relatively easy to implement scenarios on HCW availability. Figure 4, panels A and B, shows the effects of all 3 scenarios on the NEMS points availability.

Discussion

We provide calculations, which incorporate HCW absenteeism, for the surge capacity in HCWs in case of an influenza pandemic. In our model, we have shown that business continuity is maintainable when strict, clear, and disciplined hierarchical structures are in place.

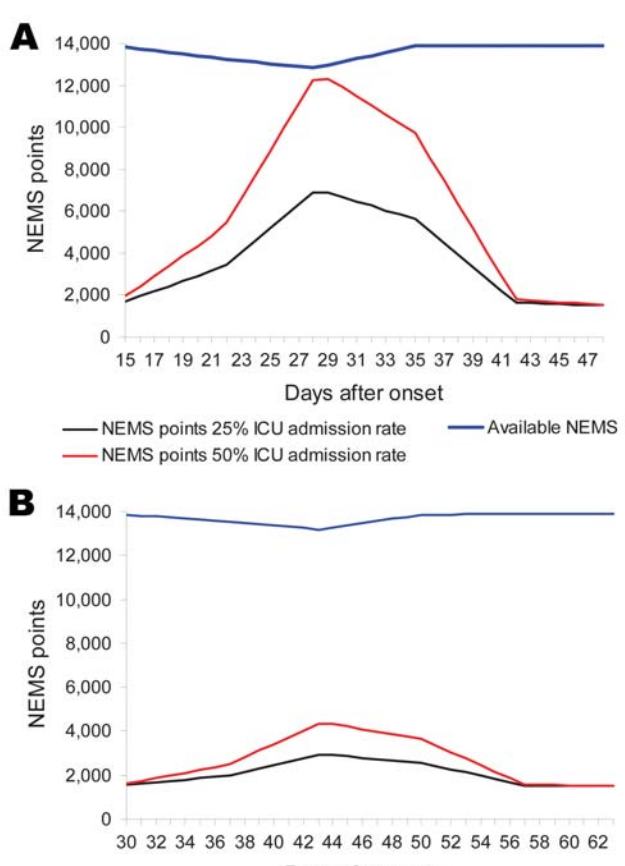

Figure 1. A) Amount of Nine Equivalents of Nursing Manpower use (NEMS) points needed and available by 25% and 50% admission rate in the intensive care unit (ICU) scenario 1, expanding healthcare worker (HCW) work hours from 8 to 12 h per 24 h (pandemic period 9 wk). B) Amount of NEMS points needed and available by 25% and 50% admission rate in ICU scenario 1, expanding HCW work hours from 8 to 12 h per 24 h (pandemic period 14 wk).

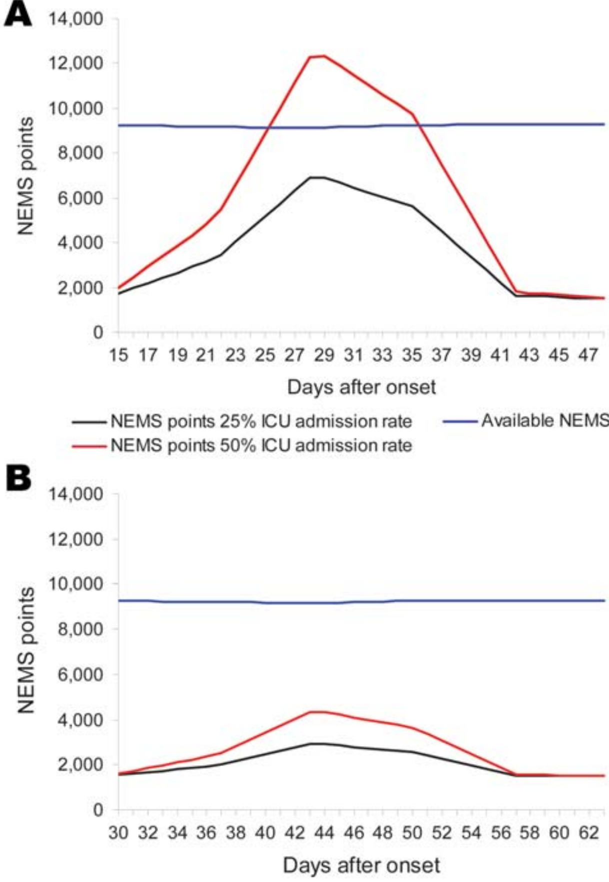

Figure 2. A) Amount of Nine Equivalents of Nursing Manpower use (NEMS) points needed and available by 25% and 50% admission rate in the intensive care unit (ICU) scenario 2, healthcare worker (HCW) 8 wk prophylactic use of neuraminidase inhibitors (pandemic period 9 wk). B) Amount of NEMS points needed and available by 25% and 50% admission rate in the ICU scenario 2, HCW 8 wk prophylactic use of neuraminidase inhibitors (pandemic period 14 wk).

Recommendation 1

Start preparations in time. When WHO declares phase IV, stop admitting all general patients, clear hospital beds, and free up HCWs. If hospitals are not cleared from responsibility for postoperative and general medical patients and if admitting general patients is not stopped, not only will there not be enough hospital and ICU beds available for ILI patients but also unnecessary risk for exposure to ILI will be placed on these patients. This step is mandatory in all hospital preparedness planning.

Recommendation 2

Implement a simple nursing workload measurement system for scarce resources. This will provide valuable information on the current workload and will aid in planning for a pandemic (*23–26*).

Recommendation 3

Gain insight, per hospital, about general and acute-care populations. This will provide information about the number of hospital and ICU beds and the number of HCWs capable of being cleared for work with ILI patients.

Length of stay for individual ICU patients is the predominant factor in capacity planning for a pandemic. We show in our results, for a worst case scenario of patients kept in ICU for 15 days, that demand for workload and ICU beds exceeds capacity (*3*). Reducing the length of stay of some patients will increase the capability of hospitals to serve most ILI patients. Therefore, we propose that a strong distinction be made between cure and care. During a pandemic, elderly patients with severe co-existing conditions may opt for supportive care without hospital and/or ICU admission, in consultation with their loved ones and their general practitioners. These patients may die because of ILI.

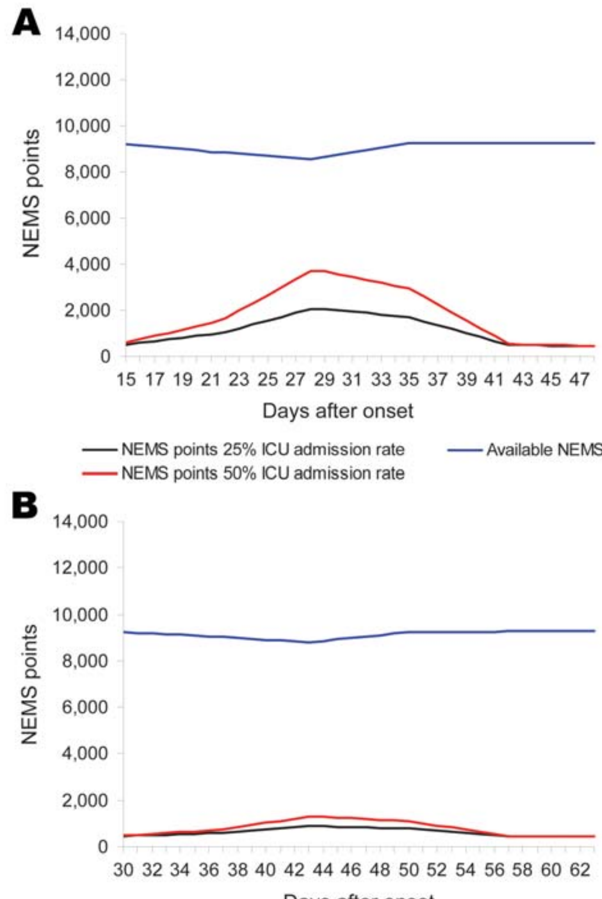

Figure 3. A) Amount of Nine Equivalents of Nursing Manpower use (NEMS) points needed and available by 25% and 50% admission rate in intensive-care unit (ICU) scenario 3, task differentiation in healthcare workers (HCWs) (pandemic period 9 wk). B) Amount of NEMS points needed and available by 25% and 50% admission rate in ICU scenario 3, task differentiation in HCWs (pandemic period 14 wk).

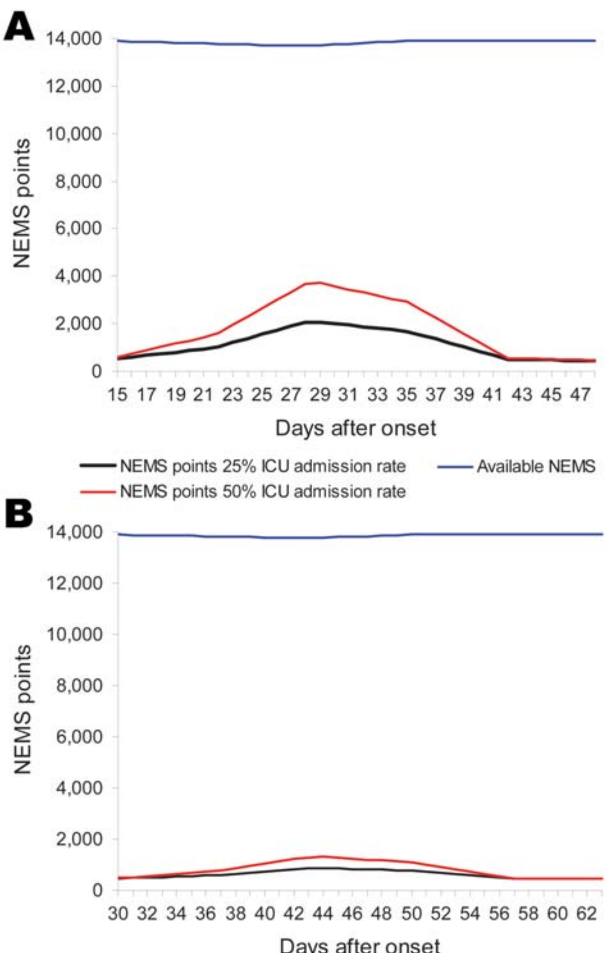

Figure 4. A) Amount of Nine Equivalents of Nursing Manpower use (NEMS) points needed and available by 25% and 50% admission rate in intensive care unit (ICU) scenarios 1, 2, and 3, combined effect of all 3 scenarios (pandemic period 9 wk). B: Amount of NEMS points needed and available by 25% and 50% admission rate in ICU scenario 1, 2, and 3, combined effect of all 3 scenarios (pandemic period 14 wk)

The smallest gain in available NEMS points occurred with scenario 2, i.e., 8 weeks' prophylactic use of neuraminidase inhibitors for HCWs. Although prophylactic use of neuraminidase inhibitors serves an import role in staff protection (*27*) and will most likely enhance HCW compliance, it serves its role specifically at the individual HCW level instead of at the workload level. The largest effect on availability of NEMS points is with scenario 3. Through insight about workload of ICU nurses and other HCWs, rigorous task differentiation can be obtained, and even specific tasks can be delegated to non-HCW specialists (for example, communication with family members of deceased patients can be done by hospital spokespersons and communications experts).

Furthermore, if all general medical and surgical patients are cleared from all hospitals in the northern part of the Netherlands, 932 medical specialists (anesthesiologists, surgeons, internal medicine, cardiologists, and cardiothoracic surgeons) can be used to provide care for acute-care and influenza patients. This strategy would greatly enhance the number of HCWs for pandemic influenza per hospital.

Finally, UMCG has 2,470 undergraduate and graduate medical students, 367 dentistry students, and 423 students' behavioral and social sciences in 2006. There are also 1,240 nursing science students and 2,391 health science students at the Hanze University Groningen of Applied Sciences. If indeed 44.3% (*28*) (226 of 510 medical students) of healthcare-related students were to report for duty in case of a HCW shortage, 3,053 extra HCWs could be recruited for duty during a pandemic to fill the potential gaps in healthcare delivery. These students would be distributed among all 15 hospitals in the region, giving each ≈203 extra HCWs.

There are several limitations to our analysis. The report by the Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza (*29*) prompts us to discuss the impact these findings have on preparedness planning of healthcare organizations. The case-fatality rate of 61% in total, especially among persons 10 to 19 years of age, and the much lower rate in persons ≥50 years of age are different from the reported rates of past pandemics (*30–32*). This increased case-fatality rate in the 10- to 19-year age group will have a tremendous effect on pandemic preparedness planning and the model presented here for hospital and healthcare institutions. Until now, we assumed persons 10–19 years of age made up only a small percentage of potential hospitalized persons (*3*). The question that has not been answered by the Writing Committee is whether this age group's particular risk is because this age group is mainly responsible for handling poultry and poultry products in reports on avian influenza. In the Netherlands, there are ≈2 million persons (12%) in this age category among the country's 16.4 million inhabitants. The same proportion holds for the 27 countries of the European Union (494 million inhabitants, 57 million persons 10–19 years of age [11.7%]).

In affluent countries, preparation for a pandemic is mostly supported and financed at the national level, and the overall belief is that hospital and intensive-care capacity will suffice. Less affluent countries might have more difficulties with pandemic preparation. The Writing Committee does not provide guidelines for preparation. For affluent countries, the economic ramifications of "losing" the younger generation struck by avian influenza might be even more dramatic then a pandemic itself because affluent countries are dealing with an increasing older population and lower birth rates.

The model used also has several limitations. The datasets of the EURICUS projects were constructed almost a decade ago. Because of changes in ICU technology and ICU nursing, decreasing emphasis on technical procedures, increased emphasis on communication within the ICU team, and communication with the patients' relatives and loved ones, some variables like NEMS (change in workload), length of stay, and ICU mortality rate (change in technical procedures) may have changed. We expect that our model presents a worst-case scenario for workload. In addition, our basic model is based on incomplete and sometimes conflicting or inconsistent information on the effects of an influenza pandemic. We assume that more reliable data will only become available in the early stages of a pandemic.

Rigorous task differentiation, clear hierarchical management, unambiguous communication, and discipline are essential. We recommend informing and training non-ICU HCWs for possible duties at the ICU. Training should address HCW needs as well as those of family and loved ones of patients with ILI admitted to the ICU. It should also incorporate the potential difficulties for HCWs in communicating with family members and loved ones if patients die after intensified treatment decisions (*3,33,34*).

Acknowledgments

We thank all the colleagues from the Groningen Regional Influenza Pandemic Working Group for helpful discussions on national and local preparedness planning for influenza pandemic situation.

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Mr Nap is pursuing a PhD degree in hospital and intensive-care capacity planning, including for infectious diseases surge capacity. His research interests include infectious disease epidemiology, disasters, and application of mathematical modeling to hospital and intensive-care resource planning.

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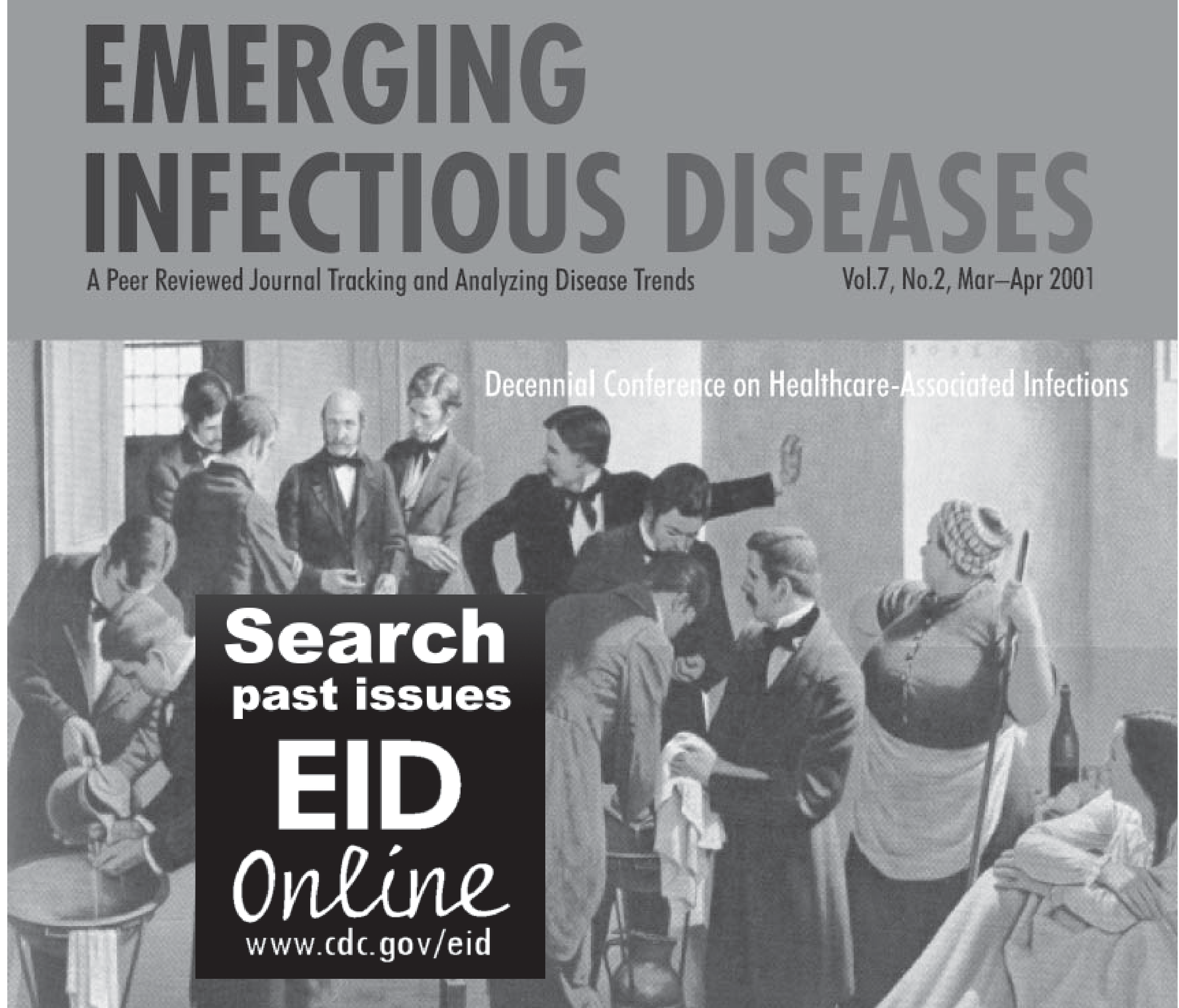

Risk Factors for Nipah Virus Encephalitis in Bangladesh[1]

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Nipah virus (NiV) is a paramyxovirus that causes severe encephalitis in humans. During January 2004, twelve patients with NiV encephalitis (NiVE) were identified in west-central Bangladesh. A case–control study was conducted to identify factors associated with NiV infection. NiVE patients from the outbreak were enrolled in a matched case-control study. Exact odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using a matched analysis. Climbing trees (83% of cases vs. 51% of controls, OR 8.2, 95% CI 1.25–∞) and contact with another NiVE patient (67% of cases vs. 9% of controls, OR 21.4, 95% CI 2.78–966.1) were associated with infection. We did not identify an increased risk for NiV infection among persons who had contact with a potential intermediate host. Although we cannot rule out person-to-person transmission, case-patients were likely infected from contact with fruit bats or their secretions.

Henipaviruses (family *Paromyxoviridae*, genus *Henipavirus*) are enveloped RNA viruses that cause respiratory illness in pigs and horses and respiratory illness and encephalitis in humans (*1–6*). After a 4- to 18-day incubation period, human disease can rapidly progress from mild illness (fever, headache, myalgia) to coma and death within 10 days; the case-fatality ratio is 40%–76% (*3*,*7–10*). The first recognized human Henipavirus infections occurred in 1994 in Australia, where a respiratory disease among horses was associated with illness in 2 humans (*11*). The etiologic agent, Hendra virus, was subsequently isolated from asymptomatic flying foxes (fruit bats of the family *Pteropodidae*) (*12*). Field et al. (*2*) suggested that horses, identified as the intermediate hosts linked to human illness, may have become infected through indirect contact with fruit bats (e.g., infected fetal bat tissues or fluids).

The first reported human epidemic of encephalitis caused by another Henipavirus, Nipah virus (NiV), occurred between September 1998 and April 1999 in Malaysia and Singapore and was associated with an outbreak of severe respiratory illness in pigs (*13–15*). Most (86%–93%) human NiV encephalitis (NiVE) infections during this outbreak involved occupational exposure to pigs, implicating these animals as an intermediate host for NiV (*15–18*). Outbreaks of NiVE occurred in Bangladesh during 2001 and 2003, in areas where NiV antibody–positive fruit bats have been identified (*19*). These reports, in addition to ecologic surveys conducted in Cambodia, have strengthened evidence that pteropid bats are the reservoir for Hendra and Nipah viruses (*12*,*20–25*).

An outbreak of encephalitis in Bangladesh was recognized on January 21, 2004; it affected 2 villages of Goalando township, Rajbari District, Dhaka Division, 70 km west of the city of Dhaka (Figure 1). Ten deaths were reported among 12 ill persons with symptoms compatible with NiVE, resulting in a case-fatality ratio of 83% (*9*,*23*). Although previous outbreaks of NiVE outside Bangladesh involved primarily men and women >25 years of age

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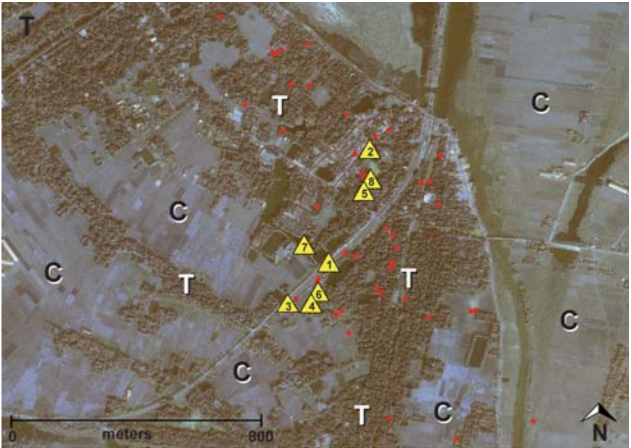

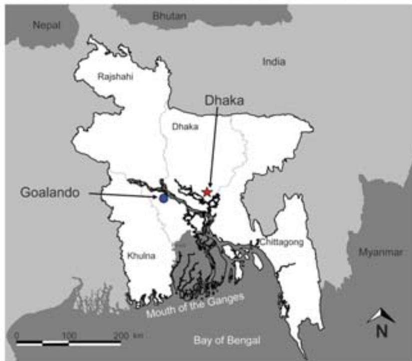

Figure 1. Top: Distribution of Nipah virus case (n = 12) and control (n = 36) households within the outbreak/study site of Goalando township, Bangladesh, January 2004. Number in the yellow triangle corresponds to household no. in Figure 2. Map also shows extreme habitat disturbance; areas under cultivation (for rice, sugar cane) are highlighted with "C," and remaining trees (fruit trees and bamboo stands) with "T." Bottom: Location of outbreak village.

(*5*,*16*,*17*,*19*,*26*), most (75%) patients in this outbreak were boys <15 years of age. We describe a matched case-control study that was conducted to characterize the epidemiology of NiVE and, specifically, to determine if risk for NiVE was associated with contact with animals; an environmental exposure, activity, or behavior; or contact with other NiVE patients during the 2004 NiVE outbreak in Goalando township.

Study Participants, Materials, and Methods

A matched case-control study was conducted in Goalando, Bangladesh (Figure 1), February 18–22, 2004. Hypotheses tested in this study, as mentioned above (e.g., increased risk for NiV infection caused by contact with animals, environmental exposure, contact with fruit in season) were based upon factors associated with previous outbreaks of NiVE in Malaysia, Singapore, and Bangladesh.

Case Definition

A confirmed NiVE case-patient was defined as any patient with fever and symptoms compatible with encephalitis after December 15, 2003, with NiV-specific immunoglobulin M antibodies in cerebrospinal fluid (CSF) or serum by enzyme immunoassay (EIA). A probable case of NiVE was defined as a patient with a diagnosis of encephalitis in whom fever developed and who was living in the same village as a patient with a confirmed case of NiVE after December 15, 2003. Cases remained in the probable category if the patient died and a specimen for laboratory confirmation could not be obtained.

We conducted a population census of the affected area in February 2004; this census was the basis for selecting controls. We identified 3 controls for each case-patient. The controls were selected randomly from the population and then matched to each case-patient on the basis of gender and age group. All households identified during the census, including houses of case-patients and controls, were mapped by Global Positioning System, and data were uploaded into ERDAS Imagine 8.5 (Leica Geosystems, Atlanta, GA, USA) and merged with a November 2000 IKONOS Geo 1-m satellite image of the outbreak area (Space Imaging, Thornton, CO, USA).

Participation was strictly voluntary, and written informed consent was obtained for all participants; for those <18 years of age, individual and parental consent was obtained. The Bangladesh Ministry of Health and Family Welfare that requested this investigation reviewed and approved all protocols.

Study Population

Probable and confirmed cases identified in 2 contiguous villages of Goalando township (Figure 1) were included in this study. Seven of the 12 cases were clustered within 3 households. Of these 7 clustered cases, 3 occurred in 1 household, and the remaining 4 were distributed in 2 separate homes (Figures 1, 2). Therefore, we conducted 2 separate analyses to assess the effect of case clustering on results. The first analysis contained the complete dataset of 12 cases and 36 controls; the subanalysis consisted of 8 cases (we randomly selected 1 case/household) and 24 matched controls. Similar results (proportions, odds ratios [ORs], 95% confidence intervals [CIs]) were obtained from both analyses. Thus, data presented in this article, including all tables, are derived from the complete dataset.

Specimen Collection and Testing

Serum samples and CSF were tested as previously described (*27*). When possible, a serum specimen was collected from controls.

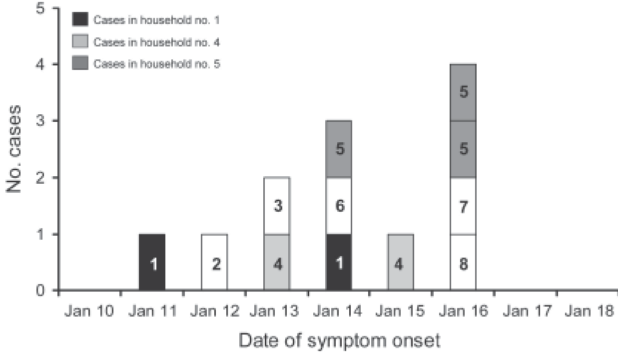

Figure 2. Epidemic curve of Nipah virus outbreak in Goalando, Bangladesh, in 2004, demonstrating household clustering. Households 1 and 4 each had 2 cases, household 5 had 3 cases, and all other households, single cases.

Data Collection and Interviews

After informed consent was obtained, case-patients and controls were interviewed at home by trained interviewers, in their native Bengali language, with a standardized questionnaire. Information such as demographics, types of animal exposures, environmental and occupational exposures, exposure to ill persons, and history of illness was obtained. Proxy interviews of family members and/or friends were conducted for deceased patients. To minimize interview bias, proxy interview methods were also used for all controls that were matched to deceased case-patients.

Statistical Analysis

Exact ORs and 95% CIs were calculated by using a matched univariate logistic regression analysis in SAS version 9.0 (SAS Institute Inc, Cary, NC, USA) (*28*). Associations were considered statistically significant at $p<0.05$.

Results

Descriptive Characteristics

Four (33%) cases were confirmed by EIA; the remaining 8 (67%) case-patients, from whom a diagnostic specimen was not available, were considered probable cases. Among all 13 (36%) controls who consented to blood collection, results of serologic tests for NiV-specific antibodies were negative. Furthermore, none of the controls reported having had a perceived fever or symptoms compatible with NiVE from December 15, 2003, through the week the study was conducted (February 18–22, 2004). In addition, an antibody prevalence study conducted among persons (n = 300) living in the outbreak site showed no evidence of asymptomatic or mild infection, which suggested that controls entered into the study were likely uninfected (A. Croisier, unpub. data). Proxy interviews were administered to equal proportions of case-patients (83%) and controls (Table 1). The median age of case-patients included in the study was 11.5 years (range 2–28 years); 9 (75%) were male, and 11 (91%) were ≤15 years of age (Table 1). Residences of all case-patients and controls were located within the affected villages, an area with a radius of ≈800 m (Figure 1).

Animal Exposures

In the matched case-control analysis, a greater percentage of case-patients (60%) than controls (34%) had observed or touched dead animals, although this finding was not statistically significant (Table 2). We observed no differences between case-patients and controls with respect to contact with ill animals (Table 2), including pigs, ruminants, and fruit bats. Chickens and ducks were often slaughtered for religious purposes or for consumption; however, close contact with these animals and their bodily fluids (e.g., blood, saliva) during this process was not associated with NiV infection (Table 2). None of the case-patients or controls had known contact with pigs (healthy or ill) or pig excreta (Table 2). Four (36%) of 11 case-patients and 7 (19%) of the controls observed fruit bats around their household during the night (OR 4.1, p = 0.49; Table 2). However, some proxy family members and/or friends answering on behalf of patients who had died were unable to answer specific questions (e.g., Did you observe fruit bats around your house during the night?).

Environmental and Behavioral Exposures

A greater proportion of case-patients (83%) than controls (51%) reported having climbed trees between December 15, 2003, and February 3, 2004 (OR 8.2, p = 0.025; Table 2). No statistically significant differences were observed between case-patients and controls with respect to outdoor activities such as hunting, fishing, or playing outdoor games (e.g., hide-and-seek, cricket, soccer). Eating fruit that was locally available (on trees or collected from fruit trees locally) between December and February

Table 1. Descriptive characteristics of Nipah virus case-patients and controls, Bangladesh, January 2004

Characteristic	No. (%)	
	Case-patients, n = 12	Controls, n = 36
Sex		
M	9 (75)	27 (75)
F	3 (25)	9 (25)
Age group, y		
1–5	1 (8)	3 (8)
6–10	4 (33)	12 (33)
11–15	6 (50)	18 (50)
16–20	0	0
21–25	0	0
26–30	1 (8)	3 (8)
Interview type		
Proxy	10 (83)	30 (83)
Self	2 (17)	6 (17)

was not associated with illness, regardless of how the fruit was collected (from the ground, picked from tree, from the market) (Table 2). Although a greater proportion of case-patients reported environmental exposures (drinking raw date palm sap, harvesting date palm sap, having someone in the household who collects date palm sap, or drinking sap directly from the collection vessel), these differences were not statistically significant (Table 2).

NiVE Case Exposure

There were strong associations between illness and 1) visiting a hospital and/or 2) having had contact with a probable or confirmed NiVE patient (Table 2). In one 2-case family cluster, a mother (26 years of age) and her infant son (2 years of age) both became ill and died. The child became symptomatic 2 days before the mother's illness onset (Figure 2; household 4). Among the other affected family clusters, the patients became ill within 3 days of one another (Figure 2; households 1 and 5); all persons in these 2 clusters reported a history of climbing fruit trees. There was no evidence of contact of persons between case households during their illness.

Discussion and Conclusions

In contrast to the patients in the Malaysian and Singapore outbreaks, which occurred primarily among adults, a preponderance of the NiV patients in the January/February 2004 Bangladesh outbreak were young boys. These findings, in the absence of high infection rates among adults or evidence of antibodies to NiV in the general population (investigation team, unpub. data), suggest an association between NiV infection and some childhood activity or

Table 2. Exposures and activities associated with Nipah virus infection, Bangladesh, December 2003–January 2004*

Exposure or activity	No. (%) study participants with reported exposure or activity†			
	Case-patients, n = 12	Controls, n = 36	OR (95% CI)	p value‡
Animal exposure				
Touched any ill animal§	9 (75)	31 (85)	1.8 (0.29–8.52)	0.613
Touched or observed a dead animal§	6/10 (60)	12 (34)	2.4 (0.4–616.5)	0.392
Killed any animal§	3 (25)	6 (16)	1.8 (0.2–79.51)	0.670
Other animal exposures				
Contact with animal stool	2/9 (22)	12 (35)	0.5 (0.05–3.04)	0.679
Visited a poultry farm	3 (25)	13 (37)	0.6 (0.08–3.29)	0.740
Observed fruit bats around household at night (1 mo before outbreak)	4/11 (36)	7 (19)	4.1 (0.27–261.9)	0.491
Outdoor activity				
Climbed trees	10 (83)	19 (51)	8.2 (1.25–∞)	0.025
Picked fruit from trees	8 (67)	18 (49)	3.2 (0.54–36.0)	0.262
Picked fruit from the ground	7/11 (64)	27 (74)	0.79 (0.13–6.09)	1.000
Fished	6 (50)	10 (28)	4.5 (0.69–49.7)	0.139
Hunted	2/10 (20)	10 (28)	7.3 (0.38–432.6)	0.240
Played hide and seek	8/11 (73)	21 (58)	4.3 (0.38–∞)	0.256
Played cricket	4 (33)	18 (51)	0.5 (0.09–2.76)	0.552
Played soccer	5 (42)	9 (24)	2.4 (0.44–16.9)	0.403
Exposure to human illness				
Had contact with a suspect or probable Nipah virus encephalitis case-patient	8 (67)	3 (9)	21.4 (2.78–966.1)	<0.001
Visiting a hospital	12 (100)	7 (19)	32.4 (5.18–∞)	<0.0001
Consumption of fruit				
Bananas¶	11 (92)	24 (67)	4.9 (0.61–226.7)	0.199
Buroys	7 (58)	28 (77)	0.4 (0.078–2.37)	0.433
Papaya	3 (25)	14 (40)	0.49 (0.08–2.24)	0.497
Guava	2 (17)	12 (33)	0.5 (0.05–2.70)	0.608
Sofeda	1 (8)	2 (5)	2.0 (0.03–38.4)	0.976
Kamranga	1 (8)	3 (9)	1.0 (0.006–165.9)	1.000
Other environmental exposures				
Drinking raw DPS	10/11 (91)	26 (72)	4.1 (0.47–197.0)	0.328
Harvesting DPS	3 (25)	3 (8)	3.4 (0.37–43.6)	0.365
Drinking DPS from collection vessel	5/10 (50)	12 (32)	1.7 (0.36–8.34)	0.612
Someone in household collects DPS	4 (33)	5 (15)	2.3 (0.38–13.3)	0.454

*OR, odds ratio; CI, confidence interval; DPS, date palm sap.
†Data are no. of study participants responding affirmatively/total no. responding (%) unless otherwise noted.
‡Exact method using univariate conditional logistic regression.
§Cows, horses, sheep, goats, pigs, ducks, chickens, dogs, cats, or fruit bats.
¶Fruit was obtained from a market or another person, if not picked directly from the tree or ground.

specific behavior. The odds of NiV infection were significantly elevated among persons who climbed trees, an activity observed almost exclusively among boys <15 years of age. This behavior is quite common among children because they gather fruit from trees. Therefore, these children may have had contact with partially eaten fruit from fruit bats or the secretions/excretions of these animals. Or, the children may have contacted contaminated fruit bat guano or urine in the trees. The percentages of case-patients playing hide-and-seek, hunting, and fishing—all of which were typical behaviorial traits of local boys—were not significantly different than those for controls. These activities generally occur outdoors; however, they do not place a child in direct contact with bat excretions or secretions, as may be true for tree climbing. Therefore, infection was apparently related to a specific behavior, tree climbing, rather than age or outdoor activities in general. Furthermore, although other exposures that may have placed persons in closer contact with bat secretions (e.g., collecting fruit or palm sap from trees, drinking palm sap directly from collection vessel) were observed more often among case-patients than controls, these findings were not statistically significant; perhaps because of the small sample size. Nonetheless, our findings can and have been used to help guide NiV outbreak investigations, leading investigators to similar conclusions as ours (*29*).

Fruit bats forage at night in various trees that are producing ripe fruit and often drink from palm sap collection vessels (*30*). Fruits are also a major food source for many villagers and, as a result of environmental disturbances (*31*) in the form of crop development (e.g., jute, rice, and sugar cane), the few remaining fruit trees grow only in close proximity to human dwellings (Figure 1). This in turn creates a situation in which fruit bats are forced into close proximity with humans, especially while these mammals are foraging and feeding. In addition, date palm sap is routinely collected in rural areas of Bangladesh between December and May. According to villagers, including palm sap harvesters, dead fruit bats are occasionally found in the collection vessels. Local villagers reported that they often observed fruit bats feeding from palm sap collection vessels, and some collectors place cloth over the opening of the vessel to prevent this (investigational team observation). In fact, a greater proportion of case-patients in our study collected palm sap, drank from the palm sap collection vessel, or had a family member who collected palm sap; however, these differences were not statistically significant. The power of our study to detect exposure risks was limited by the outbreak size. Therefore, until additional data are available, remaining cautious of date palm sap collection vessels, especially those visibly contaminated with fruit bat excreta or carcasses, would be prudent.

Numerous investigators have found serologic evidence suggesting that fruit bats of the genus *Pteropus* are the reservoir hosts for NiV (*23*,*24*), and there are reports of NiV isolation from bat urine (*20*,*25*) and partially eaten fruit (*20*). Unpublished laboratory data from the Bangladesh investigation have not supported the presence of an intermediate or primary reservoir host other than *P. giganteus*. Available data from this study, therefore, suggest direct transmission of NiV to humans through contact with bat secretions or excretions (saliva, urine, guano, partially eaten fruit) during fruit-tree climbing.

Although indirect contact with bats may have been the primary means of infection for this outbreak, Hsu and others (*19*) demonstrated that contact with ill cows was associated with an increased risk for NiV infection during the 2001 Bangladesh NiV outbreak. Therefore, intermediated hosts should be considered in future NiV outbreaks in Bangladesh.

In contrast to the patients in the Malaysia and Singapore outbreaks (*5*,*16*,*17*,*25*,*26*), most of the Bangladesh population (and all of the case-patients included in this study; data not shown) are practicing Muslims who do not consume pork and who avoid contact with pigs. None of the case-patients and controls in our study population reported any contact with pigs or pig excreta, so it is unlikely that these animals played a role in this outbreak.

Clustering of cases within households was a prominent feature of this outbreak (Figure 2); 1 household contained 3 case-patients, all brothers of ages 7–15 years. However, the longest estimated incubation periods (duration from symptom onset to first known exposure to a NiVE family member) within the clusters reported here were less than the currently recognized 4-day minimum (*7*). This finding suggests that the family clustering may have resulted from a common source of infection (e.g., a specific tree they climbed, fruit they consumed, or palm sap collection vessel they were in contact with) rather than person-to-person transmission. Our data also show strong associations between NiV infection and visiting a hospital. However, because the participants were asked if they had visited a hospital within a range of dates (December 15, 2003–February 3, 2004) and not a specific date, we were unable to determine if they were ill with NiV before visiting the hospital or whether they acquired their infection there. Some accounts in the literature suggest person-to-person transmission of NiV; therefore, it is plausible that someone could acquire, through contact with a patient's secretions or excretions, an NiV infection while visiting a hospital (*6*,*10*,*20*). Nevertheless, the most probable explanation for the observed association is that NiV encephalitis patients during this outbreak were severely ill, requiring hospitalization.

Although person-to-person transmission may have occurred in this outbreak, the initial infection (index case) may have occurred through contact with bat secretions rather than contact with an intermediate host. A limitation

of our study is that we were unable to identify a specific mechanism by which person-to-person transmission may have occurred. NiV has been isolated from the respiratory secretions and urine of patients in the Malaysia, Singapore, and current Bangladesh outbreaks (*3*,*8*,*32*,*33*), which suggests a potential for NiV to be transmitted from person to person. Data based upon chain-of-transmission events and clustering of cases during other 2003 and May 2004 Bangladesh outbreaks led investigators to conclude that human-to-human transmission may have occurred (*3*,*19*). Therefore, given the potential for household or nosocomial transmission, we recommend the use of personal protective equipment (i.e., gloves, masks, gowns, and eye protection); strict hand hygiene and surface disinfection during and after contact with an NiVE patient; isolation of patients with confirmed or suspected NiV infection; and proper disposal of potentially contaminated materials.

In summary, tree climbing, a behavior largely engaged in by young boys, was associated with an increased risk for NiV infection; although the exact mode of transmission is unclear. Our data do not rule out the potential for person-to-person transmission. If person-to-person transmission were extremely efficient, the conditions and population density of Bangladesh (≈1,000 persons/km^2; total population 141 million/144,000/km^2) may have resulted in a much larger outbreak. Indeed, a study among health workers in Bangladesh did not find evidence of incidental transmission to persons caring for patients hospitalized with Nipah-related illnesses (*34*). Bat-to-human was the most probable route of transmission in Goalando; however, some undetermined intermediate or incidental hosts cannot be ruled out. Periodic introductions of NiV to human populations in this region may continue to occur because of the overlapping nature of human and pteropid bat habitats. Moreover, bat–human interactions are likely to increase due to bat habitat loss because the few fruit trees that remain will likely be found in close proximity to human dwellings (Figure 1).

As a prevention measure, we recommend avoiding contact with fruit bats and their secretions/excretions. We also encourage persons to wash and/or peel fruit, in addition to washing their hands, before preparing meals or consuming fruit. Greater understanding of the relationships between pteropid fruit bats, NiV, and its transmission to humans might offer additional strategies for safe coexistence and disease prevention for Bangladesh and other countries where fruit bats reside. Finally, because the geographic range of this highly lethal pathogen may correspond to the distribution of the genus *Pteropus*, including parts of China and Australia, most of the Indian subcontinent, and Southeast Asia (*12*,*30*), factors that promote transmission from bats to humans need to be defined and the role of person-to-person transmission needs to be better characterized.

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Deforestation and Vectorial Capacity of *Anopheles gambiae* Giles Mosquitoes in Malaria Transmission, Kenya

Yaw A. Afrane, Tom J. Little, Bernard W. Lawson, Andrew K. Githeko, and Guiyun Yan

We investigated the effects of deforestation on microclimates and sporogonic development of *Plasmodium falciparum* parasites in *Anopheles gambiae* mosquitoes in an area of the western Kenyan highland prone to malaria epidemics. *An. gambiae* mosquitoes were fed with *P. falciparum*–infected blood through membrane feeders. Fed mosquitoes were placed in houses in forested and deforested areas in a highland area (1,500 m above sea level) and monitored for parasite development. Deforested sites had higher temperatures and relative humidities, and the overall infection rate of mosquitoes was increased compared with that in forested sites. Sporozoites appeared on average 1.1 days earlier in deforested areas. Vectorial capacity was estimated to be 77.7% higher in the deforested site than in the forested site. We showed that deforestation changes microclimates, leading to more rapid sporogonic development of *P. falciparum* and to a marked increase of malaria risk in the western Kenyan highland.

Land use changes in the form of deforestation and swamp cultivation have been occurring rapidly in the western Kenyan highlands because of unprecedented human demand for forest products and land for agricultural cultivation (*1*,*2*). In the East African highlands, 2.9 million hectares of forest were cleared between 1981 and 1990, representing an 8% reduction in forest cover in 1 decade (*3*). As has been observed in the Usambara Mountains of Tanzania (*4*) and in the southwestern highlands of Uganda (*5*), land use change in Kenya may have exacerbated malaria epidemics caused by *Plasmodium falciparum* parasites and its mosquito vectors *Anopheles gambiae* and *An. funestus* (*6*–*9*), although other factors may have also contributed to the surge in epidemics, including global warming (*10*,*11*), climate variability (*12*), and drug resistance (*4*,*13*). Land use change can influence malaria transmission by increasing the temperature and decreasing the humidity of vector mosquito habitats. This in turn affects biting, survival, and reproductive rates of vectors (*8*,*9*,*14*,*15*).

Temperature changes will also shorten the development time of *P. falciparum* in mosquitoes (*16*–*18*). Development of malaria parasites in mosquitoes (sporogony) involves a sequential process of developmental steps. Gametocytes ingested by anopheline mosquitoes develop into zygotes, ookinetes, oocysts, and eventually sporozoites in the salivary glands, ready for transmission to the next human host. Generally, the extrinsic incubation period of *P. falciparum* is inversely correlated with temperature. At 27°C, the extrinsic incubation period of *P. falciparum* from zygotes to sporozoites was found to be 12 days (*19*,*20*). The relationship between temperature and extrinsic incubation period is not linear because small changes at low temperatures can have proportionally large effects on parasites' extrinsic incubation period. Thus, geographic distribution of malaria is confined within climates favoring its extrinsic cycle, provided that other conditions do not limit mosquito survival (*21*). In turn, duration of the sporogonic development of malaria parasites in mosquitoes is an important component of vectorial capacity. Vectorial capacity measures the potential rate of contact between infectious vectors and susceptible hosts.

The objective of this study was to assess the effect of deforestation on the microclimate (temperature and

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humidity) of houses where mosquitoes normally reside with their parasites and to investigate how these microclimate changes affect the sporogonic development of the malaria parasite within *An. gambiae*. Humidity may not have a direct effect on the sporogonic development of malaria parasites, but it may affect mosquito survival through the combined effect of temperature and humidity (saturation deficit). We hypothesized that land use changes in the highlands alter the microclimatic conditions of vector mosquitoes and their parasites, subsequently enhancing malaria risk in the area.

Materials and Methods

Study Sites

This study was conducted at a highland site in Iguhu village (34°45′E, 0°10′N, 1,430–1,580 m above sea level) in Kakamega district in western Kenya. A lowland site in Kisian village (34°75′E, 0°10′S, 1,190 m above sea level) in Kisumu district in western Kenya was studied for comparison. The highland area of Kakamega district has 2 rainy seasons and ≈1,800 mm of rainfall per year. The long rainy season usually occurs between mid-March and May, with an average monthly rainfall 150–260 mm, and the short rainy season usually occurs between September and October, with an average monthly rainfall of 165 mm. Malaria prevalence peaks usually lag 1–2 months after the rain (*22*). The mean annual daily temperature is 20.8°C. A natural forest is located east of the study area, constituting ≈15% of the total area. The rest of the area is a continuum of forest in different states of deforestation. A forested area was defined as an area having a tree canopy cover >60%. The deforested area has canopy cover <10%. The deforested area was once forested but the forest has been cut down. The predominant malaria vector species in the area is *An. gambiae* s.s (*6*,*23*).

We also studied 1 lowland site (Kisian) as a comparison site for parasite development. Kisian (1,200 m above sea level) is flat land located on the shores of Lake Victoria where malaria transmission is perennial and malaria vectors include *An. gambiae* s. s., *An. arabiensis*, and *An. funestus* (*24*,*25*). The land cover type in Kisian is primarily farmland with little tree canopy coverage; thus, Kisian is classified as a deforested area. The average minimum and maximum temperatures during 1970–2000 were 15.0°C and 28.4°C, respectively. The average annual rainfall within this same period was ≈1,400 mm.

Selection of *P. falciparum* Gametocyte Carriers

The study population was primary school students in Iguhu. Children 5–14 years of age were recruited for this study and were screened monthly for *P. falciparum* gametocytes in their blood. The ethical review boards of the Kenya Medical Research Institute, Kenya, and the University of California, Irvine, reviewed and approved the protocol for screening of *P. falciparum* gametocyte carriers and subsequent mosquito infections. Parents or guardians, as well as the children involved in the study, signed a consent or assent form to participate in this study.

Mosquito Infection

Students (gametocyte carriers) who had >40 gametocytes/µL of blood and who consented to participate in the study were asked to donate 10 mL of blood, which was obtained by a clinician. Butterfly needles were used in drawing the blood into a heparinized tube. This blood was immediately centrifuged at 2,000 rpm for 5 min. The supernatant (serum) was discarded and replaced with human AB serum (Cambrex Bio Science, Walkersville, MD, USA). All equipment used was warmed to 37°C. After replacement of the serum, the blood was placed in warmed artificial membrane feeders. *An. gambiae* mosquitoes were placed in paper cups at a density of 60/cup and allowed to feed on the infected blood for 30 min. Fed mosquitoes were divided and placed in 3 cages (30 cm^3), which were hung in bedrooms of houses in deforested and forested areas in Iguhu and in the lowland site. Four houses were used at each site. At each mosquito feeding, 600 mosquitoes were provided an opportunity to blood feed and an average of 60–80% of mosquitoes took a blood meal. Fed mosquitoes were distributed equally in cages at the 3 sites. *An. gambiae* mosquitoes used in this experiment were obtained from the village of Iguhu but had been bred in an insectary and adapted to feed from a membrane feeder. All gametocyte donors were treated with amodiaquine by the clinicians in Iguhu Health Centre. Feeding of mosquitoes was conducted in a secure, insect-proof room at the Iguhu Health Centre. Fed mosquitoes were transported to different sites in paper cups placed in cool boxes.

Dissection for Oocysts and Sporozoites

Starting at 5 days after mosquitoes were exposed to different house environments, 30 mosquitoes from each cage at each site were dissected daily in 2% mercurochrome and examined for oocysts. The gut of each mosquito was carefully drawn out of the abdomen and observed under a light microscope. Infected mosquitoes were counted and the number was recorded; the oocyst load was expressed as number of oocysts per infected mosquito. Dissection for sporozoites started on day 9 postinfection. Salivary glands of each mosquito were taken from the thorax and crushed under a coverslip. This material was then observed under a microscope for sporozoites. This part of the study was conducted during April–November 2005.

Climate Data Collection

HOBO data loggers (Onset Computer Corporation, Bourne, MA, USA), which are devices for measuring temperature and humidity, were placed inside all experimental houses where the duration of sporogony of *P. falciparum* was measured to record temperature and relative humidity hourly. Data loggers were suspended from the roof 2 m above the ground. Outdoor temperature was recorded by using these data loggers placed in standard meteorologic boxes 2 m above the ground. All selected houses had a data logger for indoor and outdoor microclimate measurements. These data were offloaded from the data loggers by using a HOBO shuttle data transporter (Shuttle; Onset Computer Corporation) and downloaded to a computer by using BoxCar Pro version 4.0 (Onset Computer Corporation).

Statistical Analysis

Daily mean maximum, mean, and minimum temperatures were calculated from hourly temperature and humidity data recorded throughout the experimental period. Analysis of variance with repeated measures was used to compare monthly mean (based on the average per house in each site) temperatures throughout the entire study period. Analysis of variance was also used to test the effect of site (a fixed effect with 3 levels: forested, deforested, or lowland) and donor child (a random effect) on the average (per cage) time to sporozoite detection, the average (per cage) oocyst load, or the infection rate. Time to sporozoite infection was defined as the time postinfection needed for sporozoites to appear in dissected mosquitoes. Infection rate was defined as the proportion of mosquitoes containing oocysts or sporozoites per total number of mosquitoes dissected in each cage. This proportion was arcsin square root transformed before analysis. Oocyst load was defined as the average of oocyst counts in all the positive mosquito midguts. A total of 34 membrane feedings (34 gametocyte donors) were conducted; 29 feedings yielded infection rates >10% and were used in the data analysis. Analyses were performed with the JMP statistical package (*26*).

To evaluate how the time for sporozoite development affects vectorial capacity, we used the formula of MacDonald (*17*). For this analysis, vectorial capacity = $ma^2p^n/(-\ln(p))$, in which *m* is the relative density of vectors in relation to human density, *a* is the average number of persons bitten by 1 mosquito in 1 day, *p* is the proportion of vectors surviving per day, and n is the duration of sporogony in days. Therefore, vectorial capacity is the number of future infections that will arise from 1 current infective case. Vector abundance was calculated from the population dynamics data from the study area during June 2003–June 2004 (*27*), which was stratified by land use type. Parameter *a* was calculated as 2/first gonotrophic cycle duration that we estimated from the same study site (*8*), which was also stratified by land use type, assuming that double feeding is required for 1 gonotrophic cycle. Parameter *p* was estimated from results of our previous study on adult survival in the same setting, which is an approximation to observed age-dependent survival (*9*). Duration of sporogony was calculated on the basis of data from the present study.

Results

Indoor Environment

Mean indoor temperatures in experimental houses during the entire experimental period differed significantly among the 3 sites (forested area 21.5°C, deforested area 22.4°C, lowland 23.9°C; $F_{2,8}$ 4.5, $p<0.05$) (Figure 1). Post hoc contrast showed a difference between the lowland and either highland site. Indoor maximum and minimum temperatures mirrored this difference. Mean indoor humidity within experimental houses also differed significantly among the 3 sites (forested area 75.9%, deforested area

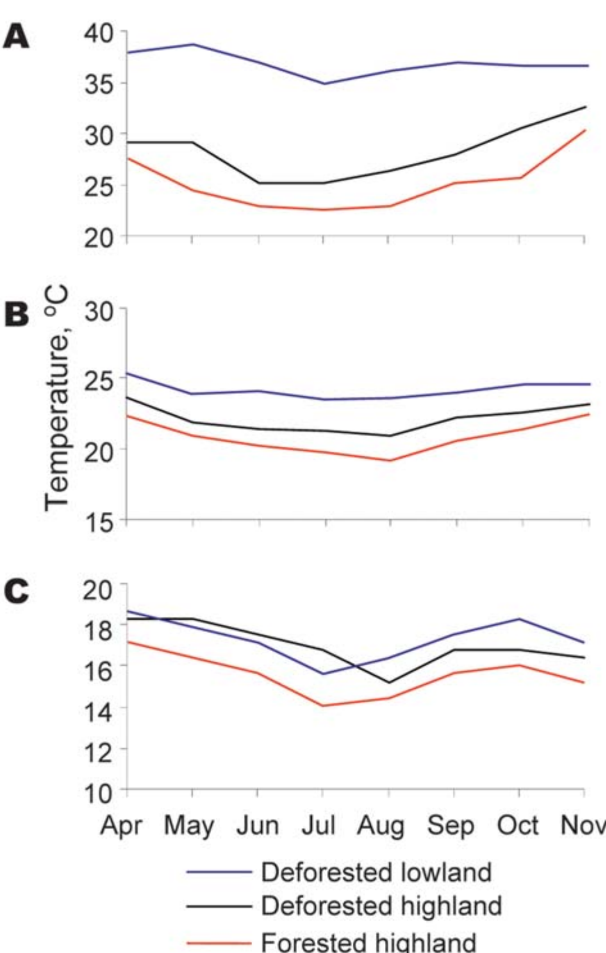

Figure 1. Monthly average value of daily maximum (A), mean (B), and minimum (C) indoor temperatures in forested and deforested areas in western Kenyan highland (Kakamega) and deforested lowland (Kisian), April–November 2005.

60.6%, lowland 64.7%; $F_{2,8}$ 8.1, $p<0.05$) (Table 1). Post hoc contrast showed a significant difference between the lowland and both highland sites and between the forested and deforested areas in the highland ($F_{1,7} = 14.5$, $p<0.001$).

Outdoor Environment

Mean outdoor temperatures within the experimental houses during the entire experimental period differed significantly among the 3 sites (forested 19.0°C, deforested 19.9°C, lowland 22.4°C; $F_{2,8} = 58.1$, $p<0.0001$). Post hoc contrast showed a significant difference between the lowland and either highland site, and also between the forested and deforested areas in the highland ($F_{1,8} = 82.8$, $p<0.05$). Indoor maximum and minimum temperatures mirrored this difference. Mean outdoor humidity did not differ among the 3 sites ($F_{2,8} = 2.6$, $p>0.05$); this was true also for maximum and minimum humidity values.

Parasite Infection and Development

The number of oocysts in a mosquito ranged from 1 to 98. The proportion of mosquitoes that carried *P. falciparum* infection (either oocysts or sporozoites) differed significantly among the sites ($F_{2,30} = 12.1$, $p<0.0001$) (Figure 2, panel A), and post hoc contrast indicated a significant forested–deforested difference within the highland site ($F_{1,30} = 16.5$, $p<0.0001$). Mean oocyst intensity was affected by land use type ($F_{2,30} = 6.5$, $p<0.01$) (Figure 2, panel B). Time for sporozoite development differed between sites ($F_{2,13} = 9.1$, $p<0.01$), and post hoc contrasts indicated a significant difference between forested and deforested areas in the highland site ($F_{1,13} = 6.9$, $p<0.05$) (Figure 2, panel C), and the lowland site exhibited the shortest sporozoite development time. Post hoc contrasts did not result in different conclusions from the alternative approach of reducing the number of factor levels in the dataset (e.g., comparing forested with deforested sites in the absence of lowland data).

Effects of Deforestation on Vectorial Capacity

Vectorial capacity at the 3 study sites was estimated by using the formula of MacDonald (*17*). Female *A. gambiae* mosquito density (*m*) was estimated to be 3.05 mosquitoes/person/day in the highland forested area, ≈4.64 in the highland deforested area, and ≈8 in the lowland site (*27*). We estimated vectorial capacity to be 77.7% higher in the deforested area than in the forested area in the highlands (Table 2).

Discussion

Environmental changes, whether natural phenomena or the result of human intervention, alter the ecologic context within which vectors and their parasites breed, develop, and transmit disease (*28*). Large-scale conversion of tropical forests for agricultural purposes can change surface properties (e.g., soil wetness, reflectivity, and evaporation rates) of an area, leading to changes in local climate (*29–31*). Bounoua et al. (*32*) reported that in the tropics and subtropics, conversion warms canopy temperature by 0.8°C year round. Our study established the role of deforestation on local microclimate and on the sporogonic development of *P. falciparum* in *An. gambiae* mosquitoes from the western Kenyan highlands. Deforestation appears to alter the climate in highlands, in particular, making it warmer and less humid. Ingestion of *P. falciparum* gametocytes by *An. gambiae* mosquitoes is more likely to result in infection, and the development time for oocyts and sporozoites is shorter in deforested sites than in forested sites. Given the established effects of temperature on *P. falciparum* development, we conclude that changes in climatic conditions caused by deforestation have caused changes in parasite infection rates and development.

Our results are supported by those of other studies. Okech et al. (*33*) showed that in different microhabitats with different temperatures in a lowland site in western

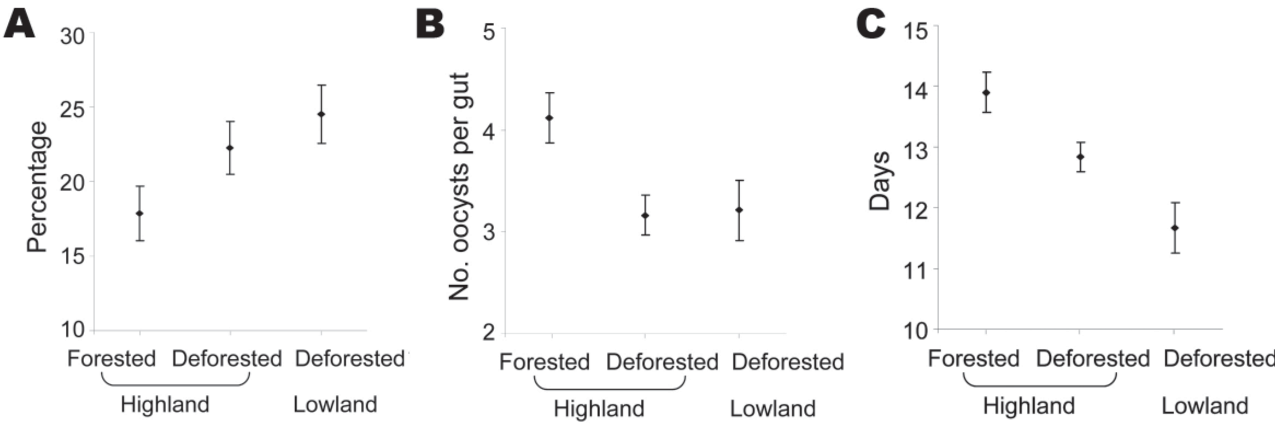

Figure 2. Mean infection rate (A), mean oocyst intensity (B), and time to sporozoite detection (C) in forested and deforested areas in western Kenyan highland (Kakamega) and deforested lowland (Kisian), April–November 2005. Error bars represent standard error.

Table 1. Climatic conditions and *Plasmodium falciparum* infection in *Anopheles gambiae* mosquitoes fed infected blood meals according to site and land use type, western Kenyan highland and lowland*

Site	Land use type	Mean ± SD indoor temperature, °C	Mean ± SD indoor relative humidity, %	No. membrane feedings	No. feedings resulting in infection	Total no. mosquitoes dissected	Range of infection rates,† %
Highland	Forested	20.89 ± 0.39[a]	75.9 ± 1.8[a]	34	27	3,171	10.0–42.8
Highland	Deforested	22.13 ± 0.27[b]	60.6 ± 2.1[b]	34	29	3,719	10.2–40.6
Lowland	Deforested	23.90 ± 0.40[c]	64.7 ± 2.1[b]	21	19	1,749	11.0–42.4

*Superscript letters after values indicate results of Tukey-type multiple comparison tests. Values with the same superscript letter in the same column were not statistically significant ($p = 0.05$).
†Infection rate refers to proportion of dissected mosquitoes that were infected with *P. falciparum* oocysts or sporozoites.

Kenya, increasing temperatures led to an increase in infection rates of *An. gambiae* mosquitoes fed on blood infected with *P. falciparum* gametocytes. However, as with our study, in the study of Okech et al. (*33*), oocyte densities did not differ with increasing temperatures. Noden et al. (*19*) examined the effect of temperature on development of *P. falciparum* in *An. stephensi* mosquitoes (normally a vector of rodent malaria) and found that the rate of ookinate development was lengthened as temperature decreased from 27°C to 21°C. They concluded that temperature affects sporogonic development of *P. falciparum* in anophelines by altering kinetics of ookinete maturation.

Our study has implications for understanding the effects of deforestation on malaria risk in the western Kenyan and other Africa highlands. Force of malaria transmission may be measured by using vectorial capacity. Duration of sporogony of *P. falciparum* in mosquitoes is exponentially related to vectorial capacity (*17*). If daily survival and biting frequency of a vector are assumed to be constant, decreasing the duration of sporogony leads to an increase in vectorial capacity. In our study, deforestation led to a decrease in duration of sporogony of *P. falciparum* by 1.1 days. Together with other factors that were also influenced by deforestation, such as increased mosquito density, biting frequency, and enhanced survivorship, This decrease translates into an increase in vectorial capacity of *An. gambiae* mosquitoes by 77.7%. The implication of this finding is that deforestation in the western Kenyan highlands could potentially increase malaria risk. In African highlands where temperature is an important driving factor for malaria and the human population generally has little functional immunity (*34*), relationships between land use, microclimate, and malaria should be carefully considered during economic development planning to mitigate the effects of malaria on human health.

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Table 2. Estimated vectorial capacity of *Anopheles gambiae* mosquitoes in forested and deforested areas, western Kenyan highland and lowland*

Site	Land use type	*m*	*a*	*N*	*P*	Vectorial capacity
Highland	Forested	3.05	0.198	13.9	0.927	0.54
Highland	Deforested	4.64	0.233	12.8	0.917	0.96
Lowland	Deforested	7.85	0.465	11.7	0.923	8.30

**m*, relative density of vectors in relation to humans; *a*, average no. children bitten by 1 mosquito in 1 day; *n*, duration of sporogony in days; *P*, proportion of vectors surviving per day. See text for details on data source and assumptions made for calculating each variable.

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Ecological Factors Associated with West Nile Virus Transmission, Northeastern United States

Heidi E. Brown,[1] James E. Childs, Maria A. Diuk-Wasser, and Durland Fish

Since 1999, West Nile virus (WNV) disease has affected the northeastern United States. To describe the spatial epidemiology and identify risk factors for disease incidence, we analyzed 8 years (1999–2006) of county-based human WNV disease surveillance data. Among the 56.6 million residents in 8 northeastern states sharing primary enzootic vectors, we found 977 cases. We controlled for population density and potential bias from surveillance and spatial proximity. Analyses demonstrated significant spatial spreading from 1999 through 2004 ($p<0.01$, $r^2 = 0.16$). A significant trend was apparent among increasingly urban counties; county quartiles with the least (<38%) forest cover had 4.4-fold greater odds (95% confidence interval [CI] 1.4–13.2, p = 0.01) of having above-median disease incidence (>0.75 cases/100,000 residents) than counties with the most (>70%) forest cover. These results quantify urbanization as a risk factor for WNV disease incidence and are consistent with knowledge of vector species in this area.

West Nile virus (WNV) disease arrived in the United States in 1999 in New York City, yet how the disease became established and details concerning the nature of the transmission cycle in the United States remain unclear. Experience in the northeastern United States suggests an urban concentration of human WNV disease cases (*1*,*2*); however, environmental factors, such as urbanization, that underlie the patterns of transmission to humans have not been explicitly evaluated. We used human surveillance data to describe and quantify the spread of WNV cases in the northeastern United States and empirically tested the hypothesis that human WNV disease is linked to the urban environment independent of human population density.

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In the northeastern United States, a mainly urban cycle of WNV transmission is supported by the role of bird and mosquito species. This enzootic cycle occurs in urban bird species; human cases occur in late summer (*2–7*). *Culex pipiens* Linnaeus is the most commonly implicated mosquito vector in the maintenance of WNV in birds (*1*,*2*,*8*,*9*). In the northeastern United States, this species feeds on birds found in urban areas, such as the American robin (*Turdus migratorius*), house sparrow (*Passer domesticus*), and European starling (*Sturnus vulgaris*) (*2*,*10*). The role of *Cx*. *pipiens* mosquitoes as primary WNV vector is supported by consistent isolations of WNV from mosquitoes captured in surveillance traps (*8*,*11–14*) and by associations between virus-infected mosquitoes and dead-bird reports (*15*).

A more contentious issue is the role of different mosquito species in transmitting, or bridging, WNV between birds and other vertebrates, including humans. *Cx. pipiens* mosquitoes are known to breed in the organically rich water of artificial containers frequently found in urban areas (*16–18*). Habitat modeling of potential WNV vectors in the northeastern United States indicates an urban focus for *Cx*. *pipiens* mosquitoes (*19*). However, its tendencies to mostly feed on birds make it an unlikely bridge vector, although other researchers have suggested that this species exhibits late season host switching to humans (*5*). *Aedes vexans* and *Cx*. *salinarius* mosquitoes have been implicated as bridge vectors in this region (*1–3*) because of their abundance and more nonspecific feeding patterns (*20*). Although both are present in urban areas, other land uses have been found to be more predictive of their distribution (*19*). These other studies do not indicate whether human incidence would be linked to the same ecological factors driving enzootic transmission.

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In this study, we explicitly tested whether both enzootic and bridge transmission occur in urban areas by evaluating human WNV disease and degree of urbanization within counties. We estimated the initial spatial spread in time to first case in Queens, New York, the site of first WNV detection (*21*), from 1999 through 2006. We also examined the trend for increasing incidence with decreasing forest cover while attempting to control for surveillance efforts and removing the effect of spatial proximity. The methods provide an example of how surveillance data with low spatial resolution can be used to quantify risk.

Methods

The study was focused in 8 northeastern states (Connecticut, Delaware, Massachusetts, Maryland, New Jersey, New York, Pennsylvania, and Rhode Island) where the same mosquito species are likely to act as primary vectors. States to the north of the study area have had limited numbers of cases and may involve different mosquito species. States farther south and west are likely to involve different species of mosquitoes; hybridization between *Cx. pipiens* and *Cx. quinquefaciatus* is more common in southern latitudes (*16*).

Human Incidence Data

We used annual numbers of human WNV cases reported to the Centers for Disease Control and Prevention (CDC) from 1999 through 2006. Human case data were acquired through multiple sources but met the CDC case definition, which includes clinical disease with laboratory confirmation. Data for 1999 were extracted from the Morbidity and Mortality Weekly Report (*22*), and data for 2000 were downloaded from the National Atlas website (http://nationalatlas.gov; *23*). Human case data for 2001 through 2006 were downloaded from the US Geological Survey maps page (http://nationalatlas.gov/printable/wnv.html; *24*). To protect anonymity, human data from these sources are compiled at the county level. All other data were aggregated by county to match this resolution.

Geographic Data

County boundaries for the United States and 2000 census data were downloaded from the National Atlas website (http://nationalatlas.gov/boundaries and http://nationalatlas.gov/people), and county centroids were identified to facilitate the calculation of distances between counties. Land-use data were downloaded by state from the US Geological Survey National Land Cover Institute (http://landcover.usgs.gov/natllandcover.php; *24*). Percentage of land cover class by county was extracted by using Fragstats Software (*25*). Land uses classified as low-intensity residential, high-intensity residential, commercial/industrial/transportation, and urban/recreational grasses were grouped into a class called *urban.* Land uses classified as deciduous, evergreen, and mixed forest were grouped into a class called *forest.* These 2 land use types were considered biologically relevant to the study question.

Statistical Analyses

To document evidence for the temporal and spatial spread of WNV disease, we generated cumulative incidence curves by state and by year and examined the distance between counties with cases. Time-to-first-case detection (in years) was used as the outcome predicted by distance to the origin, which was Queens, New York. For distance calculations, we ignored counties reporting no WNV disease cases because the first case is theoretically still to be determined. To visualize WNV disease spread, we plotted the mean incidence by year, using the spatial statistics tools of ArcGIS (*26*).

Distance measures were then used to adjust for the effect of spatial proximity in the regression analyses (*27*). Incorporating measures of spatial proximity in a regression model removes the effect of spatial structure that might otherwise result in overestimation of the strength of the association between the outcome, WNV incidence, and the explanatory environmental variables (*28*,*29*).

Logistic regression modeling was initially used to identify the relevant predictors and to quantify their relative effects by calculation of odds ratios (ORs). Number of cases per county was standardized by using the 1990 US Census population density. Cumulative WNV disease incidence data from 1999 through 2006 were dichotomized at their median to provide 2 categories of high and low risk. Predictor variables, percent urban, percent forested, county area, and per capita county income were stratified by quartiles. Logistic models were tested by using the Hosmer-Lemeshow goodness-of-fit test. The best model was selected based on the Akaike information criterion (AIC), which is a measure of fit that accounts for the number of parameters in the model. Models within 2 AIC units are considered comparable; models within 7 AIC units have less support but are still comparable; and models with differences >10 AIC units are not comparable (*30*). The relationship between increasing cases and decreasing percentage of forested land was tested by using generalized least-square regression in STATA (*31*).

A risk model of total incidence was developed by using log (count +1) transformed incidence as the response variable and the variables identified as important in the logistic regression analyses as predictors. To obtain a better fit, predictor variables were entered as continuous values for this regression. The κ statistic was used to assess agreement greater than chance between the median dichotomized original incidence and the predicted incidence, for which

<0.21 is considered slight to poor and >0.61 is considered substantial to almost perfect (*32*).

All models were initially run using only the land-use predictors; and the Moran *I* test was used to assess whether closer observations were more similar than those farther apart. This finding of an association based on spatial location could indicate that proximity, rather than environmental factors, explains the distribution of disease incidence. Distance variables control for this potential spatial proximity effect and reflect the presumed biological relationships within the data.

The models were also adjusted for surveillance effort. Human disease surveillance data must be interpreted with knowledge of the biases inherent to its collection (*33*). County per capita income was used as a measure of potential investment in surveillance and laboratory testing, as has been used in prior studies of surveillance for animal rabies (*34*).

Results

The Epidemic

From 1999 through 2006, the 204 counties in the 8 states reported 977 WNV disease cases (county mean 4.8, SD 8.7, median 1, range 0–49) (Table 1). The median county incidence over the 8-year interval was 0.75 cases/100,000 residents (mean 1.77, SD 3.0, range 0–20.2/100,000). The median incidence, excluding counties with no reported cases, was 1.70/100,000 residents (mean 2.94, SD 3.45, range 0.22–20.2/100,000) (Figure 1). The highest incidence occurred in Forest County, (20.2/100,000), followed by Cameron County (16.8/100,000) and Adams County (15.3/100,000), all rural counties in central Pennsylvania with very few cases (Forest County n = 1, Cameron County n = 1, and Adams County n = 14 [13 in 2003, 1 in 2004]), and small populations, probably representing data outliers.

Associations Based on Spatial Proximity

A cursory examination of the epidemic curve of WNV cases reported from each state during the 8-year study indicated that peak incidence was broadly overlapping in all states (Figure 2, panel A). However, cumulative distribution functions of total WNV cases (Figure 2, panel B) by year indicated that New York experienced its median case earlier in the regional epidemic than did other states (Massachusetts, New Jersey, and Connecticut), which suggests a spatiotemporal spread of WNV. Because a spatial component to spread was evident, we evaluated distance between counties to assess the spatial relationship between counties and to control for the effect of spatial proximity. The spatial component alone explained 15% of the variance in time to first case when Queens, New York, was used as the origin (n = 123 counties with cases reported, p = 0.001). After 2004, no new counties reported WNV cases, and the incidence centroids of cases in 2005 and 2006 were close to one another and had shifted back toward the origin, which suggests that the disease may have reached endemicity in the region (Figure 3).

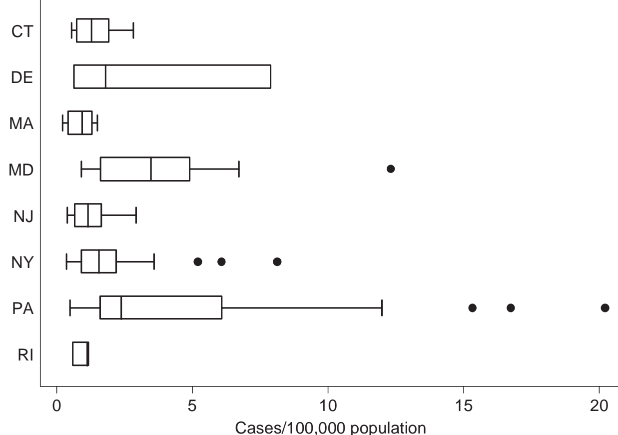

Figure 1. Box plot of total incidence of West Nile virus disease in humans, by county, for the 8 northeastern states in the study area (CT, Connecticut; DE, Delaware; MA, Massachusetts; MD, Maryland; NJ, New Jersey; NY, New York; PA, Pennsylvania; RI, Rhode Island). The box plot provides the median, lower, and upper quartiles; the standard deviation; and any data outliers. This plot excludes those counties that did not report cases. The outliers tend to be the few cases that occurred in areas with low populations.

Table 1. Incidence (per 100,000 persons) of West Nile virus disease in humans, northeastern United States, 1999–2006*

State	1999	2000	2001	2002	2003	2004	2005	2006	Mean	Median	25% IQR	75% IQR
CT	0	0.11	0.70	1.97	5.15	0.11	0.7	1.06				
DE	0	0	0	0.79	8.55	0	0.99	0	3.44	1.80	0.64	7.90
MA	0	0	0.44	2.57	2.19	0	0.61	0.27	0.43	0.11	0	0.93
MD	0	0	0.8	9.52	32.01	11.88	1.32	1.69	2.38	1.47	0	3.90
NJ	0	1.02	2.04	7.31	10.04	0.2	0.85	0.68	1.05	0.99	0.43	1.56
NY	3.18	2.45	1.19	21.03	18.78	2.44	2.95	2.03	0.87	0	0	1.25
PA	0	0	0.81	15.87	163.75	7.23	8.36	3.63	2.98	1.59	0	3.09
RI	0	0	0	0.16	2.57	0	0.16	0	0.58	0.60	0	1.13
Total	3.18	3.58	6.01	59.22	243.04	21.76	15.93	9.37	1.77	0.75	0	2.06

*IQR, interquartile range; CT, Connecticut; DE, Delaware; MA, Massachusetts; MD, Maryland; NJ, New Jersey; NY, New York; PA, Pennsylvania; RI, Rhode Island.

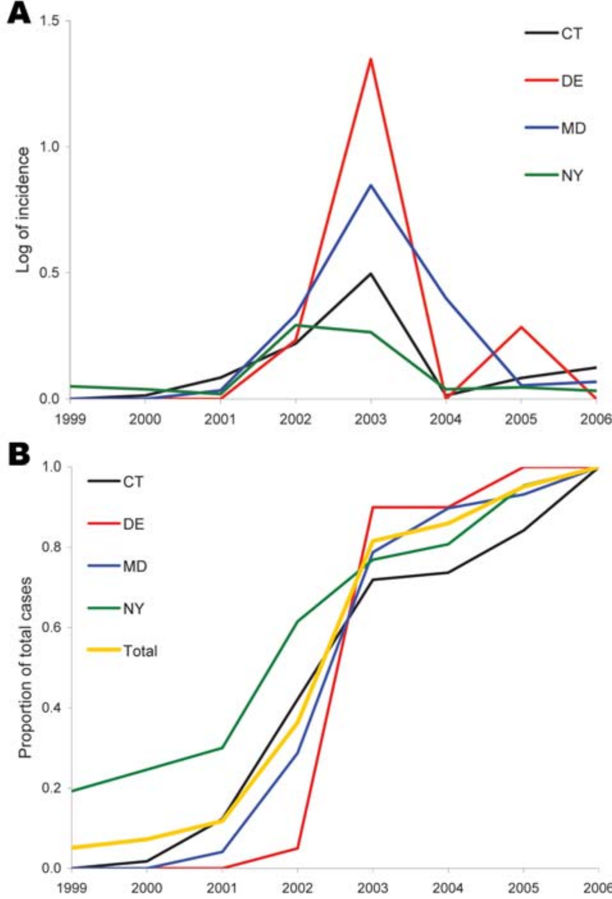

Figure 2. A) Epidemic curve of mean incidence (log+1 transformed) of West Nile virus disease in humans, by state, 1999–2006. The 4 states depicted are representative of the variation among the 8 states in the study area. CT, Connecticut; DE, Delaware; MD, Maryland; NY, New York. This graph shows the trend toward increasing incidence and a regional peak in 2003. NY seems to show a 2-year plateau with similar values for 2002 and 2003. B) Cumulative proportion of total cases for the 8 years also highlighting the 2003 regional peak but suggesting a spatial spread where cases started to rise earlier in NY than in states such as DE that were more distant from the epicenter.

Environmental Risk Factors

Risk (high or low) for WNV cases was significantly associated (by county quartile) with measures of urbanization and with percentage of forested or urban land. Because these 2 measures were highly correlated, we used only a single measure in the final analysis (Table 2). Total county area and other demographic indices (age) were not significant predictors and are not shown.

A logistic regression of the median split for total incidence with categorical predictor variables of percentage forested area and county-based per capita income showed that percentage of forested land (χ^2 = 26.13, df = 6, p<0.001) and percentage of urban land (χ^2 = 5.62, df = 6, p = 0.02) were both significant predictors of incidence (Table 2). Both models provided a good fit (forested: Pearson χ^2 = 7.82, df = 9, p = 0.55; urban: Pearson χ^2 = 3.26, df = 8, p = 0.92). No effect of spatial proximity was found among the residuals for either model (forested: Moran I = –0.008, Z = –0.49, p = 0.31; urban: Moran I = –0.002, Z = 0.40, p = 0.34).

To adjust for surveillance bias and the spatial relationship among proximal counties, we included the variables of county-based per capita income and distance from Queens, New York, respectively (Table 2). Both forested (χ^2 = 36.67, df = 11, p<0.001) and urban (χ^2 = 33.55, df = 11, p<0.001) predictors were significantly associated with WNV incidence and provided a good fit (forested: Pearson χ^2 = 209.27, df = 192, p = 0.19; urban: Pearson χ^2 = 202.78, df = 192, p = 0.28). As before, no effect of spatial proximity was found in the residuals (forested: Moran I = –0.007, Z = –0.38, p = 0.35; urban: Moran I = 0.001, Z = 0.93, p = 0.18). Although all models were significant and fit the data, the latter model was preferred on the basis of AIC (not controlling for spatial proximity $AIC_{forested}$ = 270.7, AIC_{urban} = 281.2; controlling for spatial proximity $AIC_{forested}$ = 264.1, AIC_{urban} = 267.3) and included biologically relevant controls for the effect that spatial proximity might have in estimating the association between the outcome, disease incidence, and environmental variables of interest. A general, dose-dependent trend indicated increasing incidence as measures of urbanization increased (higher incidence with decreasing percentage of pixels classified as forest in each county: χ^2 = 9.47, df = 1, p<0.01; goodness of fit χ^2 = 3.50,

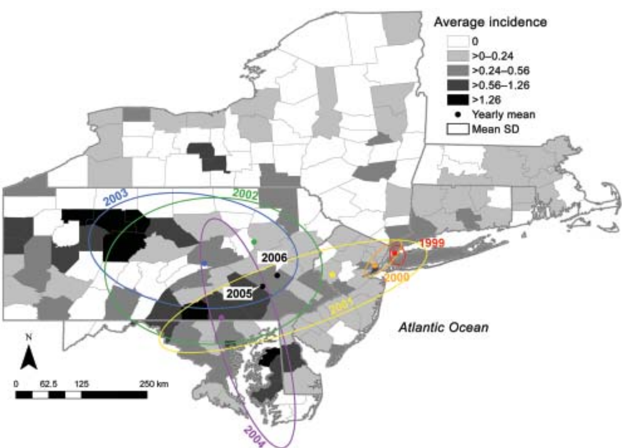

Figure 3. Incidence of human West Nile virus disease cases in 8 northeastern states, 1999–2006. Deviation ellipses indicate 1 SD of the geographic mean yearly incidence calculated as the incidence weighted average in space for each county. Incidence is attributed to the county centroid. This graph shows the urban concentration along the Eastern Seaboard as well as the outliers in western Pennsylvania (1 case in counties with low populations). The 2005 and 2006 regression of the geographic mean incidence is also depicted.

Table 2. Odds ratios for median split incidence of West Nile virus diseases in humans, for significant variables*

Predictor	Adjusted		Unadjusted	
	OR (95% CI)	Significance	OR (95% CI)	Significance
% Forest land use, per quartile				
1st (<38.29)	4.40 (1.91–10.11)	0.000	4.36 (1.44–13.25)	0.009
2nd (38.29–56.56)	3.09 (1.38–6.92)	0.006	2.86 (1.01–8.06)	0.047
3rd (56.56–69.59)	0.84 (0.37–1.91)	0.675	0.81 (0.33–2.00)	0.644
4th (>69.59)	1	NA	1	NA
% Urban land use, per quartile				
1st (<1.68)	1	NA	1	NA
2nd (1.68–4.66)	1.52 (0.68–3.39)	0.309	1.42 (0.54–3.76)	0.478
3rd (4.66–15.13)	2.44 (1.09–5.43)	0.030	3.08 (0.94–10.12)	0.064
4th (>15.13)	4.38 (1.91–10.03)	0.000	7.02 (1.78–27.71)	0.031

*Variables categorized by percent of county classified as forested and percent of county classified as urban. Outcome categorized by median split to counties with low risk (incidence <0.75 cases/100,000 residents) and high risk (incidence >0.75 cases/100,000 residents). Overall trend is for increasing incidence with increasing measures of urbanization (for decreasing percentage forested land: χ^2 = 9.47, df = 1, p< 0.01, goodness of fit χ^2 = 3.50, df = 2, p = 0.17; for increasing percentage urban land: χ^2 = 7.13, df = 1, p< 0.01, goodness of fit χ^2 = 1.98, df = 2, p = 0.37). Both unadjusted and surveillance bias and spatial relationship adjusted ORs are provided. OR, odds ratio; CI, confidence interval; NA, not applicable.

df = 2, p = 0.17; higher incidence with increasing percentage urban land: χ^2 = 7.13, df = 1, p<0.01; goodness of fit χ^2 = 1.98, df = 2, p = 0.37).

The logistic regression model of dichotomized total incidence for the 8 years of the study, controlling for income (categorical variable by quartile) and for the effect of spatial proximity (distance variables), also showed a distinct trend of increasing incidence with percentage of forest cover; counties with <38% forest cover were 4.4× more likely (95% confidence interval 1.4–13.2, p = 0.01) to have high WNV incidence than were counties with >70% forest cover (Table 2).

Predictive Model

We used the predictors identified in the logistic regression analysis to develop a linear regression model to predict total incidence (log count + 1 transformed for a normal distribution), using the quartile percent forested land by county. Per capita income (as a continuous variable) was used to control for surveillance effort. This model explains 9.7% of the variance in the total incidence (log count + 1) (p<0.001); however, the residuals indicated an effect due to spatial proximity (Moran *I* = 0.0349, Z = 5.925, p<0.001). Controlling for this spatial effect and surveillance effort resulted in a better model (r^2 = 0.20, p<0.001; Moran *I* = –0.003, Z = 0.26, p = 0.40). The κ statistic indicated good agreement (κ = 0.343, SE = 0.066, Z = 5.22, p<0.001, agreement = 67.16%) between the predicted and the observed outcomes when the binomial categorization of incidence was used and resulted in 51 county incidence entries being correctly identified as being below the median and 86 being correctly identified as being above the median. Errors were primarily in the direction of predicting the incidence above the median. When surveillance and spatial proximity were controlled for, the risk for WNV disease increased by 0.25% for every 1% decrease in forest cover. For more direct comparison with the logistic regression outcome, moving from the highest category of forest cover (>69.59%) to the lowest (<38.29%), resulted in a 6.16% increased risk for WNV disease.

Discussion

This study documents the concentration of WNV cases within urban areas of the northeastern United States and provides a quantitative estimate of the effect of varying degrees of urbanization on the risk for WNV infection at the county level. Land-use data were used to ascribe degree of urbanization as a predictor for WNV disease risk; incidence models were generated, controlling for human population density, environment-based spatial associations in the predictors, and potential biases in WNV incidence reporting resulting from the unequal resource bases among counties.

Beginning in 1999, human WNV cases were reported in counties distant from Queens, New York, the presumed origin of infection. Although the epidemic initially appeared to spread in a west/southwesterly direction in the 8-state region examined, by 2005 the initial epidemic appeared to wane, and reports of disease among newly affected counties dropped to zero. The resulting incidence maps suggest a WNV disease–endemic situation in the northeastern United States. The initial spread was not continuous along neighboring counties; rather, greater incidence was seen in urban counties after controlling for human population density, surveillance bias, and the effect of spatial proximity. The best model indicates 4× the risk for disease in the counties that fall in the lowest incidence quartile of forested land compared with the highest. The predictive nature of the data is also explored with the caveat that additional predictor variables are needed; nonetheless, it indicates increasing risk for WNV disease with decreasing forested lands.

The association between urban land use and human cases indicates that urban/suburban land use enhances en-

vironmental conditions for both enzootic and bridge transmission, at least at the county level. The spatial resolution of human surveillance data did not allow for finer evaluation of within-urban associations. Brownstein et al. linked human WNV cases to greenness indices in urban areas and found an optimal vegetation index associated with higher human cases (*35*). Brown et al. found an environmental separation of bridge and enzootic vectors; bridge vectors occurred in areas with vegetation that might be associated with residential areas within a city (*36*). Finer spatial resolution human data would allow for within-county analyses that might provide better estimations of where the cases (urban, periurban) are occurring. This would improve the predictive power of land use in the models, and the better association between land use and cases might help further elucidate which mosquito species are involved as bridge vectors.

Because of the type and resolution of the data, a sample predictive model, and not a predictive map, is provided. Nonetheless, the data and analysis provided are insightful as potentially predictive models. Additional data, such as bird abundance and perhaps also mammal abundance, are needed (*37*). Because of the often strict host and habitat preferences of mosquito species, mosquito surveillance data could also improve the predictive power and validity of the model. Our best predictive model explains only 20% of the variance; additional variables such as these might improve the model because the abundance of hosts and mosquito species will have a considerable effect on WNV transmission.

Despite the reluctance to use human surveillance data for models of disease transmission (*33*), such data can provide information about spatial associations in vector-borne disease as shown here and by others (*34*,*38*,*39*). This type of human surveillance modeling provides some useful insight into the distribution of human WNV cases and supports the current understanding of the transmission cycle.

To predict WNV disease requires understanding of the factors driving both enzootic transmission and bridging to humans. Different data availability and scales are involved in studying these 2 processes. We took advantage of the national coverage of the human incidence dataset to examine the spatiotemporal spread of WNV in this region and to generate a risk model based on land use, adjusted for the effect from spatial proximity. We show that human surveillance data at the county level are consistent with the urban nature of this disease system, as has been found in studies of enzootic transmission, indicating that the 2 processes occur in or near urban areas.

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Dr Brown graduated from the Vector Ecology Laboratory at Yale University and is a researcher with the Spatial Ecology and Epidemiology Group, University of Oxford, Oxford, UK. In her research she seeks a better understanding of vector-borne disease transmission through the investigation of the relationship between vectors and the environment in which they persist.

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Deaths from Norovirus among the Elderly, England and Wales

John P. Harris, W. John Edmunds, Richard Pebody, David W. Brown, and Ben A. Lopman

The number of deaths in England and Wales associated with gastrointestinal pathogens, norovirus in particular, in persons ≥65 years was estimated for 2001–2006. Regression analysis was used to model monthly counts of gastrointestinal pathogens in fecal samples from infected patients against monthly counts of deaths from infectious and noninfectious intestinal diseases. Data came from the Office of National Statistics (death registrations from local registrars) and from the Health Protection Agency (laboratory results). Model results suggest that 20% (13.3%–26.8%) of deaths in persons ≥65 years of age caused by infectious intestinal disease other than *Clostridium difficile* were associated with norovirus infection in this period and that 13% (7.5%–18.5%) of deaths caused by noninfectious intestinal disease were associated with norovirus. An estimated 80 deaths each year in this age group may be associated with norovirus infection.

Estimating the number of deaths associated with infection is challenging. Deaths resulting from infectious diseases tend to be underreported on death certificates (*1*). Similarly, laboratory reports record the pathogens detected but rarely record long-term outcomes, such as death or other sequelae. Routine hospital admissions and discharge data only record deaths that occur in hospital, and coding may not always be complete or accurate, especially if diagnostic results are not available (*2*,*3*).

Norovirus is the most common cause of acute gastrointestinal infections and causes most reported outbreaks of gastrointestinal disease in England and Wales (*4*). Outbreaks occur more often during the winter months of October to March (*5*), but occasionally unexpectedly high activity can occur during the summer months (*6*). To date, no published data estimate the number of deaths from norovirus infections in the United Kingdom. We estimated the number of deaths associated with gastrointestinal pathogens by using previously reported methods (*1*,*2*,*7*–*9*) and, in particular, we estimated the seasonal contribution of norovirus to death in the elderly (≥65 years of age). We also tested the hypothesis that the 2002–03 norovirus season, when a novel strain emerged (*10*), was associated with more pathogenicity than were other norovirus seasons.

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Methods

Data Sources

Laboratory Reports

The Health Protection Agency collects data from laboratories around England and Wales on pathogens identified in fecal samples from infected patients with gastrointestinal symptoms (*11*). Samples come from persons in the community (taken by general practitioners), from persons involved in outbreaks (taken by Environmental Health Officers), and from hospitalized patients. Monthly counts (based on the date the specimen was taken) of positive specimens for January 2001–December 2006 were extracted for those ≥65 years of age. The organisms responsible for gastrointestinal diseases extracted for the analysis were *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Escherichia coli* (not Shiga-toxin producing), enteric adenovirus, rotavirus, astrovirus, norovirus, *Cryptosporidium* spp., and *Giardia* spp. All other intestinal parasitic diseases were grouped together as other parasites. All bacterial pathogens were grouped by genera; e.g., all *Campylobacter* spp. were grouped together.

Mortality Statistics

The Office of National Statistics (ONS) compiles mortality statistics based on death registrations from local regis-

trars in England and Wales. Cause-of-death information on death certificates is coded by ONS according to the International Classification of Diseases, 10th revision. Annually, ONS provides HPA with a file of all deaths that mention an infectious disease code. Deaths with a code for an infectious intestinal disease (ID), either as the underlying cause of death or a contributing cause of death, were extracted for 2001–2006 for persons ≥65 years of age (Table 1). We repeated the exercise for deaths that were considered to be caused by noninfectious ID. Deaths with any mention of *Clostridium difficile* were excluded from the analysis.

Table 1. International Classification of Diseases, 10th Revision, codes used for defining deaths from infectious and noninfectious causes, England and Wales, 2001–2006

Code	Diagnosis
A00	Cholera
A01	Typhoid and paratyphoid fevers
A02	Other *Salmonella* infections
A03	Shigellosis
A04	Other bacterial intestinal infections (excludes A047, *Clostridium difficile*)
A05	Other bacterial foodborne intoxications
A06	Amebiasis
A07	Other protozoal intestinal diseases
A08	Rotaviral enteritis
A09	Diarrhea and gastroenteritis of presumed infectious origin
A212*	Pulmonary tularemia
A213*	Gastrointestinal tularemia
B462*	Gastrointestinal mucormycosis
K22*	Other diseases of esophagus
K229	Disease of esophagus, unspecified
K29*	Gastritis and duodenitis
K299	Gastroduodenitis, unspecified
K31*	Other diseases of stomach and duodenum
K319	Disease of stomach and duodenum, unspecified
K521	Toxic gastroenteritis and colitis
K528	Other specified noninfective gastroenteritis and colitis
K529	Noninfective gastroenteritis and colitis, unspecified
K92*	Other diseases of digestive system
K929	Disease of digestive system, unspecified
T47*	Poison agents primarily affecting the gastrointestinal system
T478*	Poisoning by other agents primarily affecting the gastrointestinal system
T479*	Poisoning by agent primarily affecting the gastrointestinal system unspecified
Y53*	Agents primarily affecting the gastrointestinal system
Y538*	Other agents primarily affecting the gastrointestinal system
Y539*	Agent primarily affecting the gastrointestinal system, unspecified

*Although these codes were used in the search, none yielded any results for use in our dataset.

Statistical Analyses

Because most gastrointestinal pathogens are highly seasonal, we estimated the number of gastrointestinal-related deaths by regressing monthly counts of laboratory reports on monthly counts of deaths. As we could not assume monthly counts of deaths to be normally distributed, simple linear regression models were inappropriate. We modeled monthly counts of deaths as a Poisson distribution, which has properties appropriate for analysis of count data. We used generalized linear regression models, which are used to extend simple linear regression to incorporate other distributions, to model monthly deaths as a Poisson-distributed outcome of laboratory reports of gastrointestinal pathogens.

Poisson regression also assumes that the data are not overdispersed (i.e., the variance is equal to the mean). Negative binomial models relax this assumption; we also considered negative binomial models, although they gave no qualitative differences in the results. To estimate the number of deaths that may be attributed to each pathogen, all models were fitted by using STATA 10.0 (*12*).

Our approach assumed a fixed proportion of laboratory reports for each organism to deaths over the period examined. The initial model included all laboratory reports for the extracted organisms as explanatory variables (Table 2). A constant term was included in all models to account for deaths not explained by the seasonal variation in laboratory reports. Because the number of deaths reported in each year (from both infectious and noninfectious ID) exhibited an upward trend during the study period, an independent term, consisting of year and month, was fitted to the model to account for this trend. In the initial full model, monthly deaths were modeled as a function of laboratory reports for each gastrointestinal pathogen (11 terms), the linear time variable, and a constant term (Table 2). Pathogens were removed if the coefficient was negative (because that was considered not biologically plausible) or if the variable was not significant in the model ($p>0.05$) to give the most parsimonious model. Model coefficients are on the natural scale (i.e., they represent directly how many deaths are associated with each laboratory report). The number of deaths in each month was estimated by multiplying the coefficient from the regression model for the pathogen by the number of monthly laboratory reports for that pathogen. Norovirus activity is highly seasonal; therefore, the number of deaths was also calculated with the year beginning in July and ending in June.

We carried out 2 additional analyses to test whether the 2002–03 season, when a novel norovirus strain emerged, was associated with increased pathogenicity. First, we tested for an interaction between the 2002–03 season and laboratory reports of norovirus. We looked for a significant difference in the relationship between laboratory reports of norovirus and deaths in 2002–03 season compared with other seasons (i.e., effect modification). A higher coefficient would indicate higher pathogenicity in this epidemic

Table 2. Regression model results for deaths from infectious and noninfectious gastrointestinal disease in persons ≥65 years of age, England and Wales, 2001–2006

Pathogen	Initial full model		Final model	
	Coefficient*	p value†	Coefficient*	p value†
Infectious intestinal disease models				
Norovirus	0.0134	0.003	0.0174	<0.001
Astrovirus	–0.059	0.415	–	
Shigella spp.	0.103	0.528	–	
Rotavirus	–0.055	0.003	–	
Campylobacter spp.	–0.017	0.001	–	
Escherichia coli	0.067	0.612	–	
Cryptosporidium spp.	–0.151	0.122	–	
Giardia spp.	–0.165	0.209	–	
Other parasites	–0.012	0.890	–	
Salmonella spp.	–0.011	0.478	–	
Adenovirus	–0.341	0.266	–	
Time trend	1.437	<0.001	1.611	<0.001
Constant	23.11	<0.001	6.239	<0.001
Noninfectious intestinal disease models				
Norovirus	0.0134	0.011	0.0173	<0.001
Astrovirus	0.115	0.198	–	
Shigella spp.	0.240	0.221	–	
Rotavirus	–0.066	0.004	–	
Campylobacter spp.	0.001	0.903	–	
E. coli	–0.088	0.566	–	
Cryptosporidium spp.	–0.125	0.315	–	
Giardia spp.	–0.030	0.860	–	
Other parasites	–0.081	0.439	–	
Salmonella spp.	–0.039	0.050	–	
Adenovirus	0.0147	0.967	–	
Time trend	2.008	<0.001	2.488	<0.001
Constant	20.240	<0.001	11.135	<0.001

*The coefficient represents the number of deaths associated with each laboratory report for each pathogen. The constant (intercept term) indicates monthly deaths associated with other causes.
†Wald test.

season. Secondly, we calculated a ratio of deaths to laboratory reports by dividing the number of deaths with any direct mention of viral gastroenteritis on the death certificate by the number of laboratory reports of norovirus in the corresponding year.

Results

During 2001–2006, a total of 1,136 deaths were recorded with any code for infectious ID and 1,736 for noninfectious ID (Figure 1). Infectious and noninfectious ID–associated deaths were correlated ($R^2 = 0.33$, p = 0.10, Figure 1) and exhibited a wintertime seasonal pattern. Over the same period (2001–2006) in England and Wales, a total of 65,932 laboratory reports of the pathogens of interest were submitted for those ≥65 years of age. Summertime seasonality of the major bacterial pathogens and the wintertime seasonality of viral pathogens for this age group are illustrated in Figure 2, panels A–C.

Table 2 shows the comparisons of the best fitting models for infectious and noninfectious ID–associated deaths. Norovirus was the only pathogen significantly associated with monthly counts of infectious ID deaths (p<0.001). All other pathogens were removed from the model. In the noninfectious ID deaths model, astrovirus was also significant (p = 0.02). Because astrovirus infection is rare in the elderly (*4*) and shares a similar seasonality with norovirus, we decided to leave it out of the final model. A linear term, accounting for the general increasing trend in the number of deaths reported, significantly improved the model (p<0.001) and was included in the final estimation. Norovirus remained the only significant pathogen in the model. Therefore, in the final models the expected number of norovirus deaths was modeled as a Poisson distribution of laboratory reports of norovirus and a linear time variable. Some slight overdispersion was evident in the infectious ID deaths model, but fitting an alternative model to account for the overdispersion, negative binomial models, did not substantially alter the results, which suggests that Poisson regression is not inappropriate in this case.

The model estimates that during 2001–2006, a total of 228 deaths from infectious ID were associated with norovirus infection, which represents 20% (13.3%–26.8%) of deaths from infectious ID in those ≥65 years of age; 225 (13% [7.5%–18.5%]) of deaths from noninfectious ID were

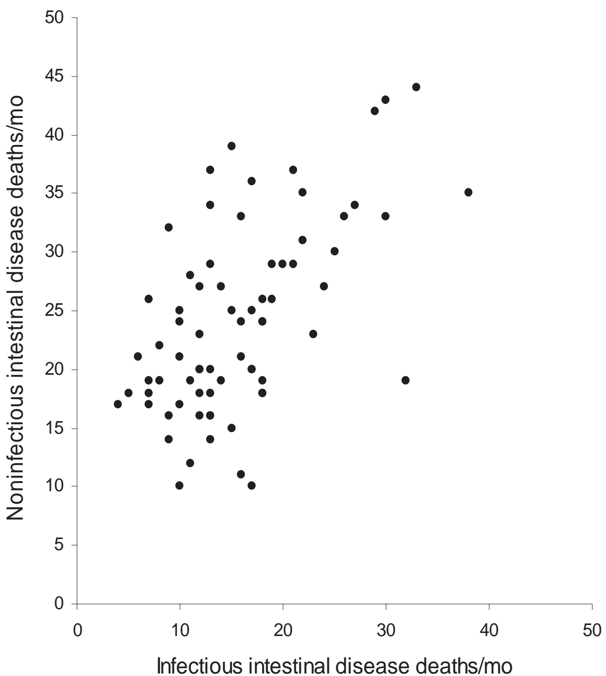


Figure 1. Correlation of monthly death reports of infectious and noninfectious intestinal disease, England and Wales, 2001–2006.

associated with norovirus. Thus, the annual average number of deaths (January to December) from both infectious ID and noninfectious ID was 38; however, when looking at the period from July to June in each year, to account for the norovirus season, the average was ≈40 each season (Table 3). Figure 3, panels A, B, illustrates that models fit better to the deaths from infectious ID but still show some association with the deaths from noninfectious ID.

In years with high seasonal activity, numbers of norovirus-associated deaths were higher. The overall death/laboratory report ratio for 2001–2006 was 55/1,000 (95% confidence interval [CI] 51–60). The ratio did not increase in the years with greater numbers of deaths (Figure 4), and we found no evidence that the ratio was significantly higher during any of the study years. The 2002–03 season had the lowest death/laboratory report ratio. Including an interaction term in the infectious ID model between the epidemic 2002–03 season and laboratory reports of norovirus resulted in a negative-coefficient interaction term (likelihood ratio test p value = 0.002). This finding suggests a lower death/laboratory report ratio in the 2002–03 season, contrary to the hypothesis we were testing. The relative risk for death in the 2002–03 season compared with all other seasons was 0.81 (95% CI 0.69–0.96, p = 0.016).

In an analysis of recorded causes for all infectious ID deaths, viral gastroenteritis was specifically listed as an underlying cause for 13.4% of deaths (152/1,136). Table 4 shows the distribution of recorded underlying causes when viral gastroenteritis was mentioned as a contributory cause in 20% (227/1,136) of infectious ID deaths. For noninfectious ID deaths, diseases of the digestive system accounted for 52% (898/1,736) of the recorded underlying causes. Of these, 92% (829/898) were caused by unspecified noninfectious gastroenteritis and colitis.

Discussion

Over the 6-year period of our study, the total number of deaths in persons ≥65 years of age that may be attributable to norovirus was 453 (228 from infectious ID and 225 from noninfectious ID). On average, this equates to ≈80 deaths each year attributable to norovirus infection. Norovirus was the only gastrointestinal pathogen that was consistently significant in the 2 regression models.

Of the recorded deaths from infectious ID, 13% had viral gastroenteritis listed as the underlying cause. For deaths from noninfectious ID, 48% had an underlying cause of unspecified noninfectious gastroenteritis and colitis. Because these are unspecified causes, and given their similar seasonality with infectious ID, many of these are likely to be infectious causes that were misclassified.

In years with higher norovirus activity, more deaths were associated with norovirus infection. However, we

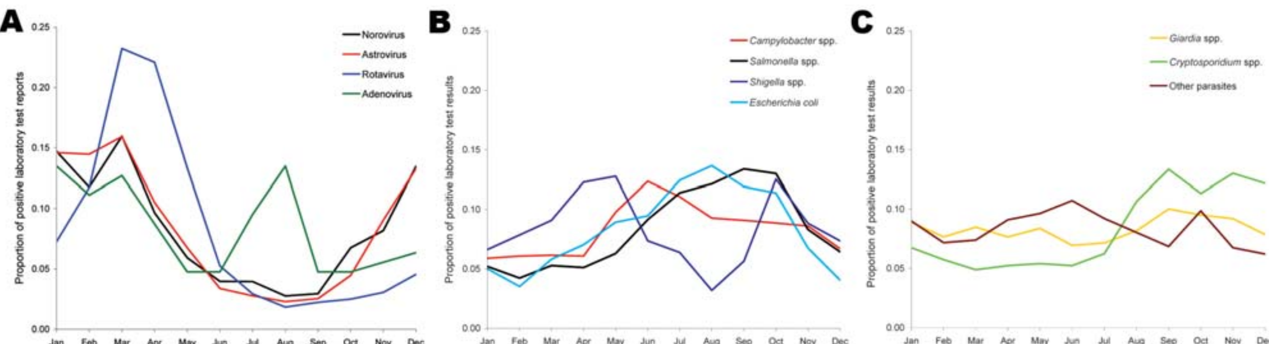
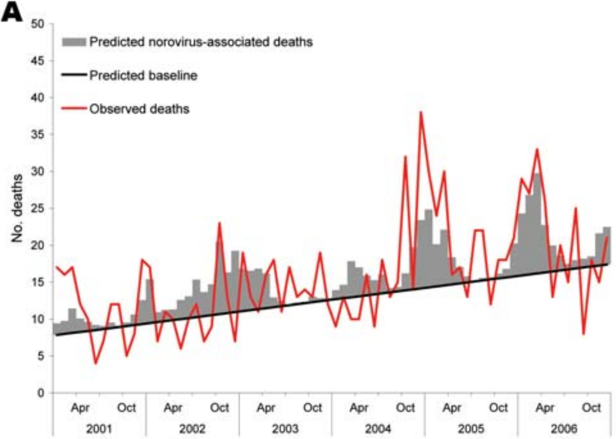


Figure 2. Seasonality of positive laboratory reports of viral (A), bacterial (B), and parasitic (C) pathogens, persons ≥65 years of age, England and Wales, 2001–2006.

Table 3. Estimated number of deaths in each season (July to June) from the regression models, England and Wales, 2001–2006

Year	Predicted annual deaths, no. (95% confidence interval)		
	Infectious intestinal disease only	Noninfectious intestinal disease only	Infectious and noninfectious intestinal disease
2001–02	22.2 (14.7–29.7)	21.9 (12.7–31.2)	44.3 (32.4–56.2)
2002–03	59.5 (39.4–79.5)	58.8 (34.0–83.7)	118.9 (86.9–150.8)
2003–04	18.4(12.2–24.6)	18.2 (10.5–25.9)	36.8 (26.9–46.7)
2004–05	51.4(34.1–68.8)	50.9 (29.4–72.4)	102.8 (75.2–130.5)
2005–06	51.8(34.3–69.3)	51.3 (29.6–72.9)	103.6 (75.7–131.4)
Total	203.3 (134.6–272.0)	201.2 (116.1–286.2)	406.5 (297.2–515.7)
Annual mean	40.7	40.2	81.3

found no evidence of increased pathogenicity in years with higher recorded norovirus activity. The season when a new variant of the genotype II.4 virus emerged (2002–03) did not coincide with an increase in death/laboratory-report ratio. Indeed, the opposite was observed; fewer deaths as a proportion of positive laboratory reports were observed, the interaction term showed a negative coefficient for that season, and the relative risk for death during that season was lower than during other seasons.



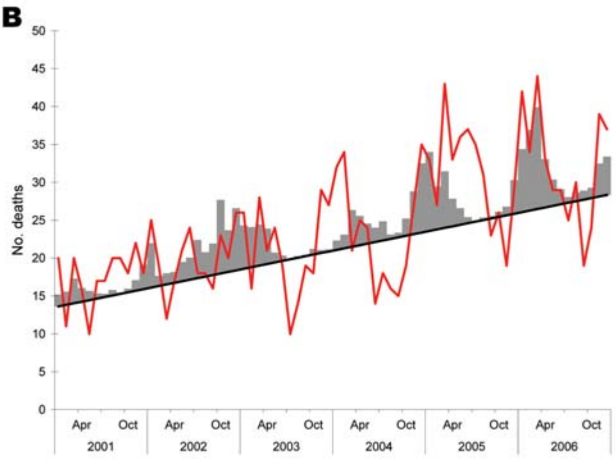


Figure 3. Observed and expected monthly deaths in persons ≥65 years of age from infectious intestinal diseases (A) and noninfectious intestinal diseases (B), derived from the most parsimonious models. England and Wales, 2001–2006.

One of the assumptions in our regression model was that laboratory reporting was consistent over the time of the study. Laboratory reporting processes did not change during the years of the study and are unlikely to have caused bias in this study. Testing and reporting behavior, however, may have changed over time. The number of specimens identified by PCR and ELISA increased from ≈70% to ≈90% in the study period. Thus, the decreased ratio of deaths/laboratory reports may have resulted from increased testing during the 2002–03 season rather than from the virus being less pathogenic during that year.

The modeling approach may underestimate the contribution of norovirus and other pathogens because the method estimates how much of the seasonal variation in death is associated with the seasonal variation in laboratory reports. Less-seasonal pathogens are less likely to show an association, and nonseasonal components (i.e., background levels) will not be attributed to a pathogen. Indeed, a substantial constant term in our models represented these unattributed deaths. The model for deaths from noninfectious ID did not appear to be as good a fit as the model for deaths from infectious ID. There was, in our opinion, enough evidence of a correlation between infectious and noninfectious ID to make a case for including this model.

This method has been used in the past for other pathogens (rotavirus, respiratory syncytial virus, pneumococcus, influenza virus) and unexplained deaths; when we used it in this study, we found an association between norovirus and death. Until our study, most reports of norovirus-associated deaths have been anecdotal (*13*). Although deaths associated with norovirus infection have been documented (*14*,*15*), these are usually singular reports of patients having died subsequent to infection with norovirus, rather than in-depth analysis of time trends of death.

In this study we attempted to go further and estimate the extent of death from norovirus. Norovirus is usually considered a mild, self-limiting disease, and most of those infected with the disease make a full recovery with no long-lasting effects. However, this study shows that part of the population, those ≥65 years of age, have a small risk of dying as a result of contracting norovirus. Rates of infection are higher within healthcare settings than in the commu-

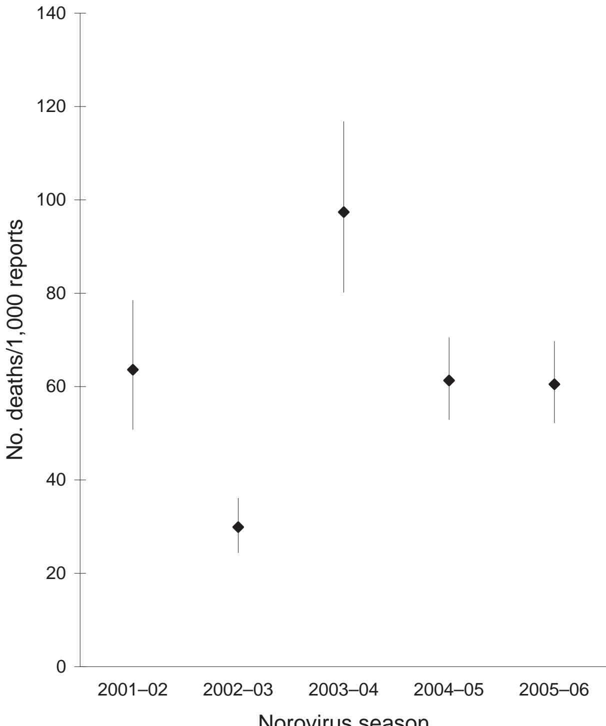


Figure 4. Ratio of viral gastroenteritis–associated death reports to norovirus laboratory reports, 5 norovirus seasons (2001–2006), England and Wales.

nity (*4*,*15*,*16*). Previous studies have shown that hospital patients who are involved in outbreaks of norovirus are ill longer than those who become infected in other settings (*15*). In England the proportion of the population ≥65 years of age is increasing. In years to come, this will be a substantial proportion of persons at risk, and deaths associated with this disease may well increase.

Noroviruses are known to evolve quickly. Emergence of new variants of the most commonly circulating strain can cause epidemic years in which more outbreaks occur and many more persons are infected. New variants are also associated with out-of-season activity, i.e., more outbreaks and infections than usual occurring in summer. When this happens, most of the population may be susceptible to infection. Our study suggests that when such epidemics occur, the number of norovirus-associated deaths increases as a result of the large number of persons infected rather than from increased virulence. Nevertheless, a measurable amount of death is associated with norovirus infection every year.

Table 4. Recorded underlying causes of death in persons in whom viral infectious intestinal disease was a contributory factor

Underlying cause of death	%
Infectious intestinal disease (viral)	40.2
Circulatory system disorder	32.8
Respiratory system disorder	9.0
Neoplasm	4.9
Digestive system disorder	2.5
Nervous system disorder	2.5
Endocrine, nutritional, and metabolic disorders	2.5
Infectious intestinal disease (bacterial)	1.6
Mental/behavioral disorder	1.6
Musculoskeletal and connective tissue disorder	1.6
Genitourinary system disorder	0.8

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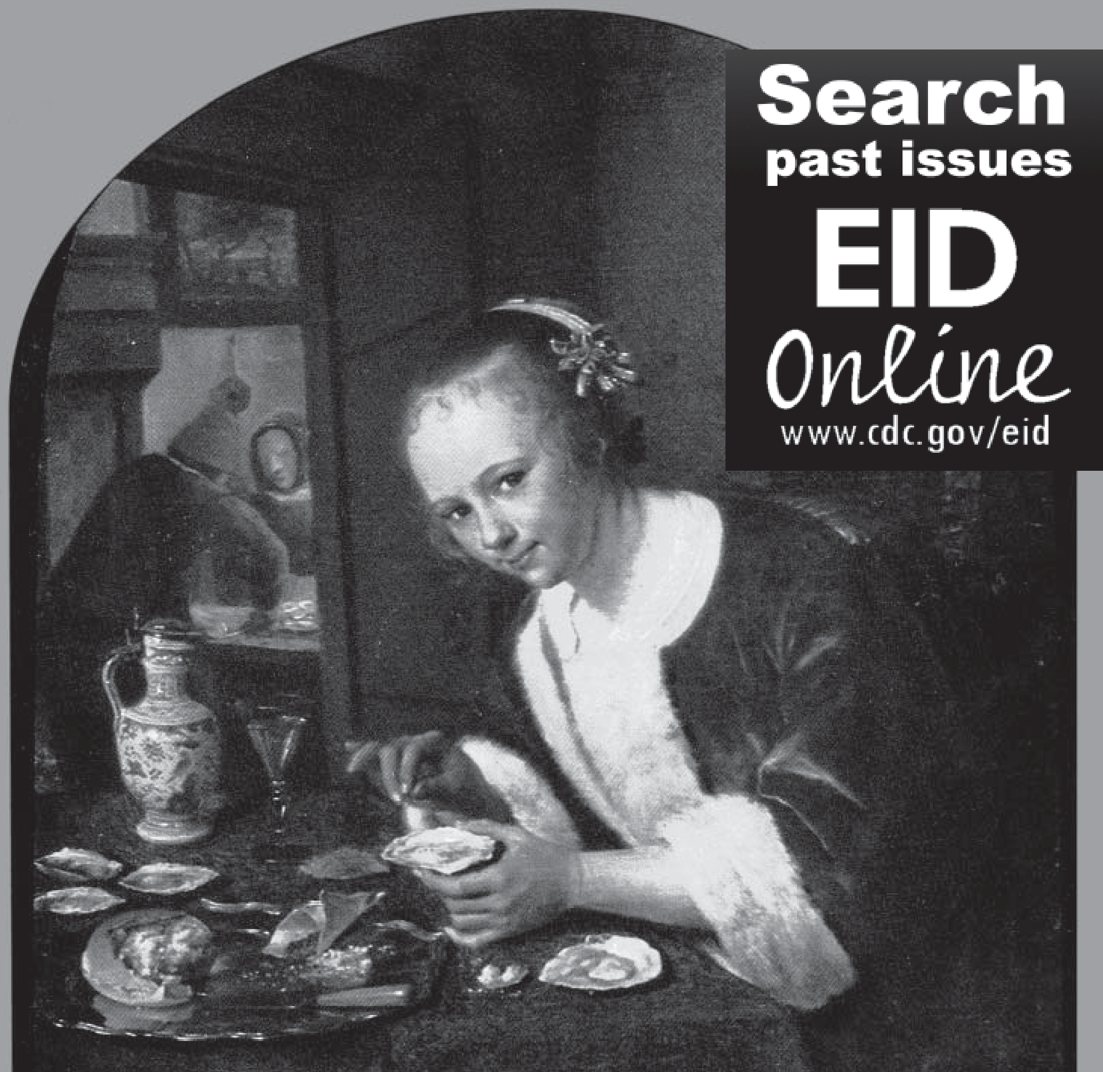

Norwalk Virus Shedding after Experimental Human Infection

Robert L. Atmar, Antone R. Opekun, Mark A. Gilger, Mary K. Estes, Sue E. Crawford, Frederick H. Neill, and David Y. Graham

Noroviruses are the most common cause of viral gastroenteritis in the United States. To determine the magnitude and duration of virus shedding in feces, we evaluated persons who had been experimentally infected with Norwalk virus. Of 16 persons, clinical gastroenteritis (watery diarrhea and/or vomiting) developed in 11; symptomatic illness lasted 1–2 days. Virus shedding was first detected by reverse transcription–PCR (RT-PCR) 18 hours after participant inoculation and lasted a median of 28 days after inoculation (range 13–56 days). The median peak amount of virus shedding was 95 × 10^9 (range 0.5–1,640 ×10^9) genomic copies/g feces as measured by quantitative RT-PCR. Virus shedding was first detected by antigen ELISA ≈33 hours (median 42 hours) after inoculation and lasted 10 days (median 7 days) after inoculation. Understanding of the relevance of prolonged fecal norovirus excretion must await the development of sensitive methods to measure virus infectivity.

Noroviruses are the most common cause of epidemic gastroenteritis in the United States (*1*). Although in vitro replication systems for these viruses have recently been described (*2*,*3*), human noroviruses cannot readily be grown in cell culture, and no small animal model of human norovirus infection is available. Much of what is known about these viruses has by necessity been learned from experimental human infection and from observational studies of naturally acquired infection. Norwalk virus is the prototype strain in the genus *Norovirus*, and many of the human experimental infection studies have used this strain (*4*–*9*). We describe the duration and magnitude of virus shedding in persons infected with Norwalk virus after experimental inoculation.

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Materials and Methods

Virus Inoculum

Liquid feces from persons who participated in a previous Norwalk virus challenge study (*8*) were screened to identify samples with high concentrations of Norwalk virus RNA (>10^7 reverse transcription–PCR [RT-PCR] U/mL). The participants were recontacted and screened for health (results within normal limits for liver function, tuberculosis skin test [negative], and chest radiographs; negative serologic test results for hepatitis A, B, and C, retroviruses [HIV-1, HIV-2, and human T-lymphotropic virus 1 and 2], and syphilis [nonreactive rapid plasma regain]). The new challenge inoculum (lot 42399) was prepared from liquid feces of 1 participant by clarification, centrifugation, and serial filtration through filters with progressively smaller pore size to a final 0.45-µm filter size. The inoculum, which contained no other enteric viruses or adventitious agents, was packaged and stored at –80°C.

Challenge Protocol

Challenge studies were conducted from September 2004 through October 2006. Healthy adults (18–50 years of age) provided informed consent and successfully completed a test of understanding. In addition, eligible persons were secretor positive (because secretor-negative persons are resistant to Norwalk virus infection; *9,10*), had screening laboratory study results that were within normal limits (liver and renal function, blood counts), had negative serologic results for hepatitis and HIV, had no serious chronic diseases, had no history of nonbacterial gastroenteritis within 3 months of inoculation or of bacterial or protozoal enteric infection within 1 month (based on 3 negative enteric cultures and fecal ova and parasite studies in the 4-week preinoculation screening period), were

not exposed to persons considered to be at risk for more severe norovirus infection (e.g., immunocompromised patients, the elderly, and children), and were not employed in jobs identified as having high risk for transmission to other persons (e.g., food service, healthcare, and airline industries). On the day of inoculation, participants were admitted to the Baylor College of Medicine General Clinical Research Center and orally received different dosages of inoculum (10-fold dilutions ranging from 4.8 to 4,800 RT-PCR units) or placebo in the early evening. Inoculated participants remained in the Center for a minimum of 96 hours and at discharge had experienced no watery feces or vomiting for at least 18 hours. Participants' clinical signs and symptoms were evaluated every 4 hours while they were in the Center, and all fecal samples were collected and refrigerated immediately. Within 24 hours of collection, the samples were transported to the laboratory for processing and stored at –70°C until analyzed. After patient discharge, all fecal samples were collected daily for 21 days and then weekly for up to 5 additional weeks (for a total observation time of up to 8 weeks postinoculation). The samples were delivered to the laboratory within a day of collection and were processed and stored as described above. Participants were educated about the importance of hand washing and hand hygiene at the beginning of the study, and these concepts were reinforced at each study visit. The clinical protocol was reviewed and approved by the Institutional Review Board at Baylor College of Medicine, and an Investigational New Drug application describing the clinical protocol and study inoculum was reviewed by the US Food and Drug Administration.

Laboratory Studies

Norwalk virus–specific antigen was detected by sandwich ELISA, using Norwalk virus–specific antiserum, as previously described (*8*), and Norwalk virus–specific antibody was detected by ELISA, using Norwalk virus–like particles as antigen, as previously described (*8*). Norwalk virus RNA was detected in fecal specimens by using either an immunomagnetic capture (IMC) RT-PCR assay (*11*) or quantitated by real-time RT-PCR (qRT-PCR) with RNA transcripts as a standard (*2*). The primers used for the IMC RT-PCR assay were the antisense Norwalk virus p35 (5′-CTT GTT GGT TTG AGG CCA TAT-3′) and the sense Norwalk virus p36 (5′-ATA AAA GTT GGC ATG AAC A-3′); probe was a 5′ digoxigenin-labeled Norwalk virus p69 (5′-GGC CTG CCA TCT GGA TTG CC-3′). For the qRT-PCR assay, a 10% fecal sample was diluted 1,000-fold and heated to 95°C for 5 min (*12*); 20 µL of heated sample was analyzed in duplicate wells. The primers for the qRT-PCR assay were the antisense Norwalk virus p165 (5′-CAT AAT CAC CTA CAT CCA TCT CAG ATG-3′, which is complementary to Norwalk virus nt 4689–4715) and the sense primer Norwalk virus p166 (5′-CGG CCT CAC CAG AAT TGG-3′, which is complementary to Norwalk virus nt 4641–4658); the probe was Norwalk virus p167 (5′-FAM/CGA GGT TGT GGC CCA AGA TTT GCT AG/TAMRA-3′, which is complementary to nt 4660–4685). For determination of a virus titer, both wells had to show amplification. The limits of detection for the IMC RT-PCR and qRT-PCR assays were ≈15 × 10^3 and ≈40 × 10^6 copies/g feces, respectively.

Definitions

Infection was defined as seroresponse (4-fold rise in titer from preinoculation baseline to 30-day serum sample, as measured by ELISA) or fecal virus excretion as detected by RT-PCR or presence of antigen. Viral gastroenteritis was defined as illness with moderate diarrhea (alone) for any continuous 24-hour period (moderate diarrhea is >200 g of watery feces that immediately take the shape of the collection container) or 1 vomiting episode plus 1 of the following: abdominal cramps or pain, nausea, bloating, loose feces (if not fulfilling the definition of diarrhea), fever (oral temperature ≥37.6°C), myalgia, or headache.

Results

A total of 16 persons inoculated with Norwalk virus met the criteria for having Norwalk virus infection. Of these, 11 (69%) met the predefined definition for viral gastroenteritis. The 5 who did not meet this predefined definition had ≥3 symptoms that did not include vomiting or >200 g of watery diarrhea. All 11 participants with viral gastroenteritis had abdominal cramps, nausea, and vomiting; 5 of these participants also had >200 g of watery diarrhea, and 1 had <200 g of watery feces. Other signs and symptoms in the 11 participants were malaise (n = 9), anorexia (n = 8), headache (n = 7), myalgia (n = 4), temperature ≥37.6°C (n = 4), and chills (n = 3). The 5 participants who did not fulfill the criteria for gastroenteritis had nausea (n = 5), anorexia (n = 5), malaise (n = 4), abdominal cramps (n = 3), myalgia (n = 3), headache (n = 3), temperature ≥37.6°C (n = 2), chills (n = 2), and watery diarrhea <200 g (n = 2). Although the number of infected participants in each dosage group was relatively small, no differences in signs and symptoms based on inoculum dosage were apparent. The median duration of signs and symptoms was 23 (range 10–61) hours and was similar for both groups of participants.

All infected participants shed virus as measured by RT-PCR and had a ≥4-fold rise in serum antibody level, and all but 2 also shed virus as measured by antigen ELISA (Table). Virus shedding as measured by IMC RT-PCR was first detected a median of 36 hours (range 18–110 hours) after inoculation and lasted a median of 28 days after inoculation (range 13–56 days). Norwalk virus was detected

Table. Fecal virus shedding among 16 participants inoculated with Norwalk virus*

Participant no.	Estimated Norwalk virus inoculum dose (RT-PCR units)	First–last study days† postinoculation when symptoms present	First–last study days IMC RT-PCR positive	Day peak virus titer (character of feces)	Peak qRT-PCR virus titer ($\log_{10}$/g)	First–last study days postinoculation when antigen positive
Met clinical definition of gastroenteritis						
Had diarrhea and vomiting						
706	4,800	2	2–28‡	2 (liquid)	11.1	2–9
707	4,800	2–4	1–30‡	2 (liquid)	9.5	4–8
710	4,800	1–2	2–30‡	5 (solid)	10.9	2–7
722	48	2	2–48	4 (solid)	11.7	2–8
724	4.8	2–3	2–56	3 (solid)	11.4	2–6
Had vomiting only						
701	4,800	1–2	1–29‡	4 (solid)	10.8	3–10
720§	48	2	2–56	4 (solid)	11.5	2–9
721	48	1–3	2–21	4 (solid)	11.7	2–10
723	48	2	1–50	4 (solid)	12.2	2–6
731	4.8	2–3	5–10	5 (solid)	10.0	None
732	4.8	2–3	2–15	3 (solid)	11.9	2–6
Median	–	2	2–30‡	4 (solid)	11.4	2–8
Did not meet clinical definition of gastroenteritis						
703	4,800	2–3	1–32‡	2 (solid)	10.7	2–9
704	4,800	2–3	4–21‡	5 (solid)	9.2	5–7
715§	48	2–3	1–28	3 (solid)	11.7	2–5
716§	48	2–3	1–20	4 (unformed)	10.1	3–7
717	48	3	4–13	4 (solid)	9.3	None
Median	–	2–3	1–21‡	4 (solid)	10.1	2–7

*RT–PCR, reverse transcription–PCR; IMC, immunomagnetic capture; qRT-PCR, quantitative PCR.
†Study days are reported as calendar days; study day 1 began ≈5–6 h postinoculation.
‡Fecal samples only collected for 30 d postinoculation.
§Watery feces with mass <200 g.

in fecal samples of 7 participants 3–14 hours before onset of any clinical signs or symptoms. Presymptomatic shedding was more common in persons who did not meet the definition of clinical gastroenteritis than in those who did (4/5 vs. 3/11, respectively, p = 0.11, 2-tailed Fisher exact test). Virus shedding as measured by antigen ELISA was first detected ≈33 hours (median 42 hours) after inoculation and was last detected 10 days (median 7 days) after inoculation. Median values of the onset and resolution of virus shedding, as measured by IMC RT-PCR or antigen ELISA, were similar for the participants who had clinical gastroenteritis compared with those of persons who did not meet the definition of gastroenteritis (Table).

Norwalk virus concentration in feces, as measured by qRT-PCR, peaked a median of 4 days after inoculation; the time of peak shedding was similar for participants who did and did not meet the definition of viral gastroenteritis (Table). The highest fecal concentrations of virus were detected in 11 (69%) participants after their clinical signs had resolved. The median peak amount of virus shedding was 95×10^9 (range $0.5–1,640 \times 10^9$) genomic copies/g feces, and 5 participants shed $\geq 100 \times 10^6$ copies/g until at least day 14 (Figure 1). Persons who met the clinical definition of gastroenteritis had a higher median peak of virus shedding than those who did not have gastroenteritis (250×10^9 vs. 12×10^9 genomic copies/g feces, p = 0.08, Wilcoxon rank sum), and the average total number of viral genomic copies measured in the feces over the first 2 weeks after inoculation also was higher in the clinical gastroenteritis group ($10^{13.3}$ vs. $10^{12.4}$, p = 0.056, Student *t* test). The virus concentrations in feces collected later after inoculation were low (range $225,000–40 \times 10^6$ genomic copies/g). Correlation between virus titer in feces and optical density results obtained in the antigen ELISA was strong (r = 0.823, Pearson correlation, p<0.001; Figure 2).

Discussion

Noroviruses are estimated to cause 23 million cases of gastroenteritis in the United States each year and to be the most common cause of foodborne gastroenteritis (*13*). Relatively few data describe the quantity and duration of fecal norovirus shedding as determined by modern assays. In a human experimental Norwalk virus infection model, we found that Norwalk virus could be detected in fecal samples for a median of 4 weeks and for up to 8 weeks after virus inoculation and that peak virus titers were most commonly found in fecal samples collected after resolution of symptoms. The peak virus titers (median 95×10^9 copies/g feces) were higher than would be expected from electron microscopic studies (*5*,*14*). These observations help explain the epidemiologic observations of norovirus outbreaks linked to food handlers who had recovered from

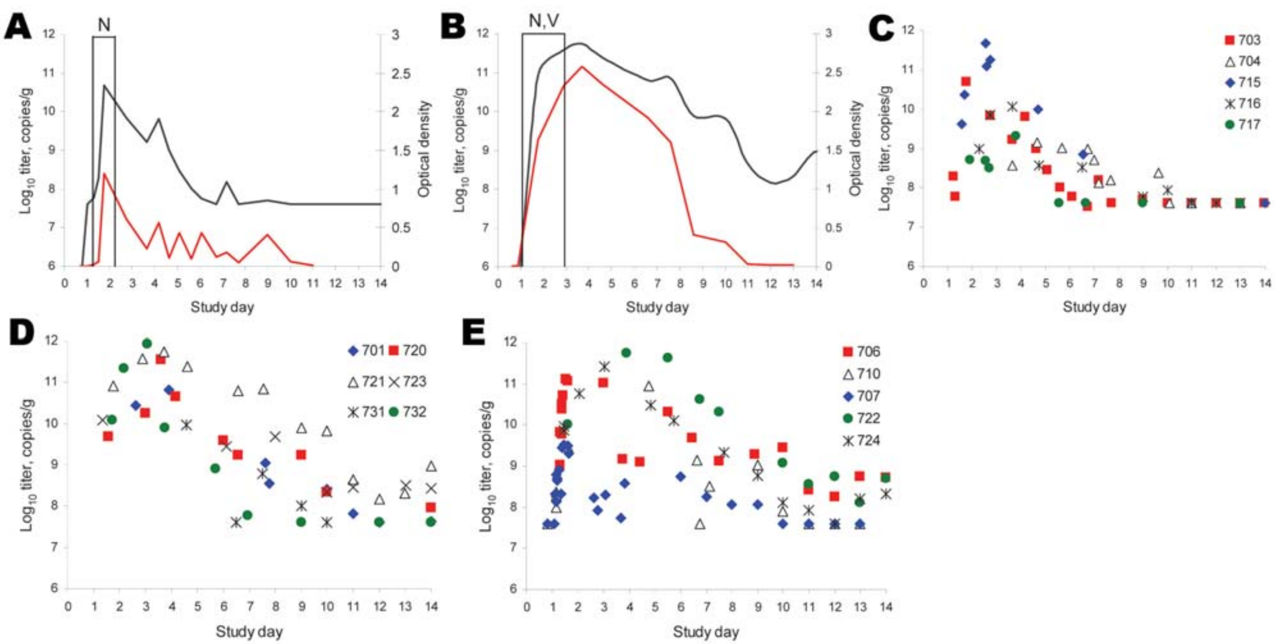

Figure 1. Shedding of Norwalk virus in feces. The quantity of viral RNA measured by quantitative reverse transcription–PCR (qRT-PCR; black line) and of virus antigen measured by ELISA (optical density; blue line) is shown for 2 participants: no. 703, who did not have clinical gastroenteritis (panel A), and no. 721, who had clinical gastroenteritis (panel B). Vertical lines represent the period of clinical symptoms; N, nausea; V, vomiting. Panels C, D, and E show the virus titers as measured by qRT-PCR in fecal samples collected from participants who had no clinical gastroenteritis, had gastroenteritis with vomiting only, and had gastroenteritis with vomiting and diarrhea, respectively.

symptomatic infection (*15*) and in persons who had no gastroenteritis (*16*).

Only a few studies have used quantitative RT-PCRs to examine fecal viral load, and these studies have been primarily of GII norovirus strains. Chan et al. (*17*) described patients who shed >10^{11} norovirus copies/g feces, whereas

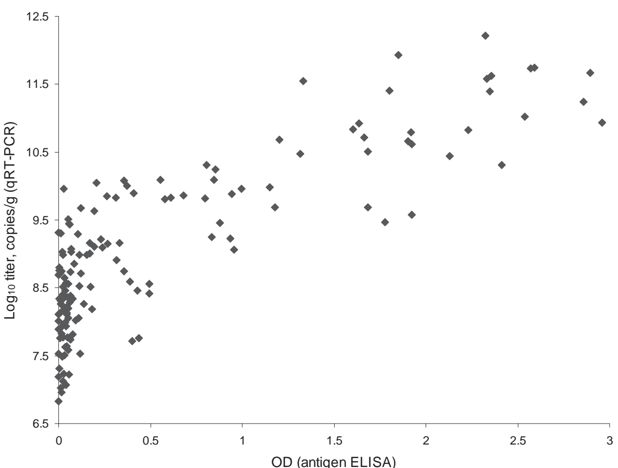

Figure 2. Correlation of viral RNA titer with antigen ELISA (optical density [OD]). Titers of viral RNA are correlated with the OD measured by antigen ELISA for the 148 fecal samples with positive quantitative reverse transcription–PCR (qRT-PCR) results (r^2 = 0.823, Spearman correlation, p<0.001).

the peak fecal virus titer observed by Ozawa et al. (*18*) in symptomatic and asymptomatic food handlers was ≈10-fold lower. Each of these studies was of persons with naturally acquired norovirus infection. However, the median peak viral load observed in our study (10^{11}) was much higher than the 10^7–10^8 median viral loads reported in the prior studies (*17*,*18*). Lee et al. (*19*) noted higher viral loads in patients who had more prolonged symptoms (≥4 days) associated with infection caused by GII.4 norovirus. Amar et al. (*20*) also reported viral loads to be higher in persons who had symptomatic gastroenteritis than in those who had been asymptomatic for at least 3 weeks. Our findings suggest that clinical gastroenteritis was associated with higher peak virus shedding and higher total virus shedding during the first 2 weeks after inoculation. Although we did not see an association of peak virus titer with symptom duration, the median duration of symptoms averaged only ≈1 day in our study. Potential reasons for the different results observed in other studies include the manner in which samples were collected (single samples vs. serial collection), the real-time assays used (generic assays designed to be broadly reactive vs. assay designed specifically for Norwalk virus detection), virulence of the infecting strains, differences in the populations studied (e.g., age, immune status), and the small number of infected persons who did not have clinical gastroenteritis in our study.

The development of more sensitive methods to detect noroviruses has been associated with a corresponding increase in the duration of recognized virus shedding (*1*,*8*). For example, Rockx et al. (*21*) found norovirus in fecal samples for ≥3 weeks in ≈25% of infected persons, and Murata et al. (*22*) found norovirus in fecal samples for up to 6 weeks in infected infants. In contrast, at least half of the participants in our study still had Norwalk virus in fecal samples after 4 weeks and 2 had virus still present at 8 weeks; we cannot exclude the possibility that these 2 persons shed for a longer period. Determination of whether the virus is still infectious must await the development of more sensitive and reproducible methods for norovirus cultivation than are currently available (*23*).

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Prophylaxis after Exposure to *Coxiella burnetii*

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Coxiella burnetii is a category B bioterrorism agent. We numerically evaluated the risks and benefits from postexposure prophylaxis (PEP) after an intentional release of *C. burnetii* to the general population, pregnant women, and other high-risk populations. For each group, we constructed a decision tree to estimate illness and deaths averted by use of PEP/100,000 population. We calculated the threshold points at which the number of PEP-related adverse events was equal to the cases averted. PEP was defined as doxycycline (100 mg 2×/day for 5 days), except for pregnant women, where we assumed a PEP of trimethoprim-sulfamethoxazole (160 mg/800 mg 2×/day) for the duration of the pregnancy. PEP would begin 8–12 days postexposure. On the basis of upper-bound probability estimates of PEP-related adverse events for doxycycline, we concluded that the risk for Q fever illness outweighs the risk for antimicrobial drug–related adverse events when the probability of *C. burnetii* exposure is ≥7% (pregnant women using trimethoprim-sulfamethoxazole = 16%).

Q fever is caused by the intracellular bacterium *Coxiella burnetii* and is endemic in nearly every country in the world. A zoonotic disease, it is usually transmitted to humans through aerosolization of the bacteria from animal products; person-to-person transmission is rare (*1–4*).

Roughly 50% of all *C. burnetii* human infections are asymptomatic (*5–8*). Acute illness is usually characterized by sudden onset febrile illness; chronic disease occurs in ≈1% of all acute cases with endocarditis being the most common chronic condition (60%–70%) (*1,8–12*). Persons with preexisting cardiac valve defects are at significantly higher risk for chronic disease; chronic disease develops in 39% of patients treated for acute disease (in 75% without treatment) (*13–15*). Immunocompromised patients (e.g., HIV-positive and cancer patients) are also at increased risk for chronic illness.

A Q fever–associated chronic fatigue syndrome may exist as well. Although prevalence is controversial, studies have cited that 10%–30% of all patients with acute disease report persistent symptoms (e.g., fatigue, myalgia, night sweats) more than a year after acute infection occurred (*10,16*). Pregnant women are also at increased risk for severe acute *C. burnetii* infection because of the bacterium's predilection for the placenta. Premature birth (33%) and spontaneous abortion/neonatal deaths (39%) occur frequently in acutely ill pregnant women (*17*).

C. burnetii is classified as a category B bioterrorism agent by the Centers for Disease Control and Prevention and the National Center for Allergy and Infectious Diseases (*18*). Regardless of the likelihood that *C. burnetii* may be used as a bioterrorism agent due to its status as a category B agent, public health agencies are obligated to prepare for such a scenario. Current Q fever postexposure prophylaxis (PEP) guidelines for the general population are 100 mg of doxycycline (or 500 mg tetracycline 2×/day for 5 days), started 8–12 days postexposure (*4*). This recommendation is based on limited studies conducted at Fort Detrick, Maryland, USA in the 1950s, which indicated that administering antimicrobial drugs directly after exposure to *C. burnetii* extended the incubation period by 8–10 days but did not prevent infection from occurring (*19*). Waiting 8–12 days after exposure before starting treatment prevented illness (*19*). Unfortunately, these guidelines do not account for the probability of exposure and prophylaxis-related adverse events. Also, the US government has not published any PEP recommendations for pregnant women, although trimethoprim-sulfamethoxazole (TMP-SMX) has been suggested as a possibility (*1,4*).

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To assist in the development of PEP recommendations, we present a risk-benefit analysis, estimating the number of cases of illness/death that could be averted with PEP after a large release of Q fever versus a treatment-only strategy where antimicrobial drugs are administered only upon symptom onset. We also determine the threshold probability of exposure at which the risk for antimicrobial-related adverse events outweighs the risk for Q fever illness.

Methods

Model

In 2006, we conducted a risk-benefit analysis for each of the following groups: the general population, high-risk populations (persons with valvular defects or heart problems and the immunocompromised), and pregnant women. Total medical outcomes averted for each group were calculated by using the following general equation:

> Total medical cases averted = (Total adverse health outcomes caused by Q fever without PEP) – (total adverse health outcomes caused by Q fever remaining after intervention) – (cases of PEP-related adverse events)

The online Technical Appendix (available from www.cdc.gov/EID/content/14/10/1558-Techapp.pdf) contains the equations defining each input (e.g., total adverse outcomes without PEP) of this equation.

To calculate adverse outcomes with and without PEP, we constructed a decision tree for each target group illustrating all possible outcomes after exposure to *C. burnetii*. The general population and high-risk populations share the same tree structure (Figures 1, 2); the tree for pregnant women incorporates the outcomes for the unborn child (Figure 3). Drug-related side effects are not included in Figures 1–3; however, the number of side effects was calculated per Equation 4 in the online Technical Appendix. Total medical cases averted were calculated at 4 arbitrary levels of *C. burnetii* exposure (100%, 50%, 25%, and 10%).

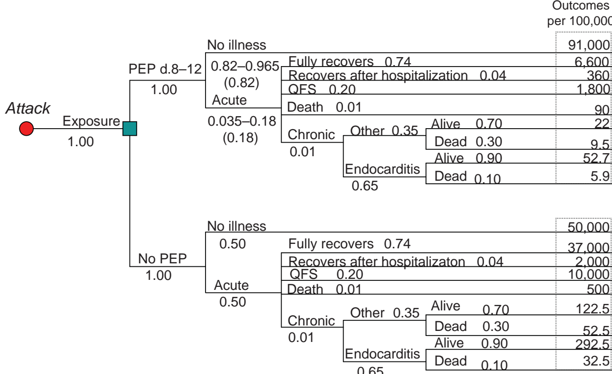
Cohort Size and Discounting

We assumed a cohort of 100,000 for each model. Also, given that each exposed patient would begin to fully experience any adverse health outcome from either Q fever or PEP within 1 year, we did not discount outcomes.

Assumptions

Several assumptions were made in conducting this risk-benefit analysis. For simplicity's sake, 100% compliance was assumed for persons receiving PEP. Risk-benefit analyses are based on an aerosolized, point source, overt attack, for which response can begin almost immediately. Estimates of cases and cases averted are based on the assumption that persons in whom acute or chronic illness develops receive appropriate treatment and care once a diagnosis of Q fever has been made. Those exposed received the same dose of *C. burnetii*. Although limited studies have shown an increase in dose can decrease the incubation period of the disease and/or increase the severity of illness, we were concerned with preventing illness all together (*9,20*).

Because Q fever has a low infectious dose (a single spore/bacterium may be enough to cause illness) (*19*), we assumed any dose would be sufficient to cause clinical infection. PEP does not affect the course or severity of illness in persons who become ill after having received prophylactic antibimicrobial drugs (persons in the PEP and no-PEP groups have the same probability of outcome events occurring once acute illness developed); persons in the No Illness group are assumed to have no latent illness.



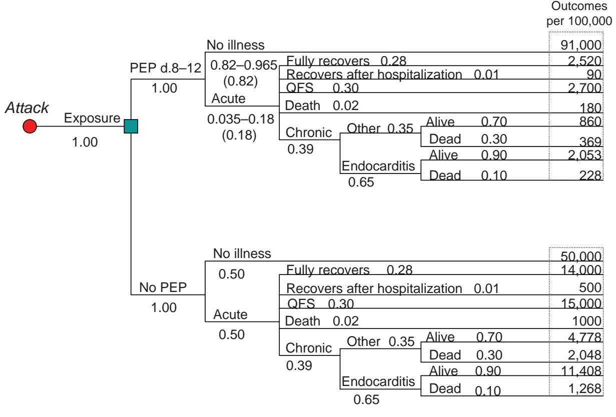
No. (%) of population with specific outcome by PEP use

	Total illness	Severe illness	Death
No PEP	50,000 (50)	13,000 (13)	585 (5.9)
With PEP	9,000 (9.0)	2,340 (2.3)	105 (0.11)

Figure 1. Decision tree for a general population of 100,000 based on an assumption of an aerosolized, point source, overt attack with *Coxiella burnetii* (postexposure prophylaxis [PEP] with 100 mg doxycycline 2×/d for 5 d, assuming 82% drug efficacy and 100% exposure). PEP-related adverse events are not included in this figure. The probability of each individual event occurring is provided in the decision tree under the respective event title (i.e., 1.00 for Exposure). Some events list a range of probabilities with the specific probability for this scenario in parentheses (i.e., 0.82–0.965 (0.82) for PEP No illness). The number of persons with each respective outcome is listed on the right side of the tree. A summary of outcomes (total illness, severe illness, and death) and the percentage of the population with such an outcome are provided in the table below the PEP and No PEP trees. We defined total illness as all acute illness, severe illness, and Q fever–related deaths. Severe illness was defined as hospitalization during acute infection, chronic illness, Q fever fatigue syndrome (QFS), or death. This description also applies to Figures 2 and 3.

Interventions

Our analyses considered 2 different PEP options. For the general and high-risk populations, we assumed a PEP



No. (%) of population with specific outcome by PEP use

	Total illness	Severe illness	Death
No PEP	50,000 (50)	36,000 (36)	4,315 (4.3)
With PEP	9,000 (9.0)	6,480 (6.5)	777 (0.78)

Figure 2. Decision tree for a high-risk population of 100,000 based on an assumption of an aerosolized, point source, overt attack with *Coxiella burnetii* (postexposure prophylaxis [PEP] with 100 mg doxycycline 2×/d for 5 d, assuming 82% drug efficacy and 100% exposure). PEP-related adverse events are not included in this figure. QFS, Q fever fatigue syndrome.

dosage of 100 mg of doxycycline 2×/day for 5 days, beginning 8–12 days postexposure. As doxycycline is generally not recommended for pregnant women, we assumed a PEP dosage of 160 mg/800 mg TMP-SMX 2×/day for the duration of the pregnancy, starting 8–12 days postexposure (*21*).

Q Fever–related Outcomes

To provide some sense of risk-by-severity of outcome, we categorized health outcomes into 3 cumulative categories: total illness, severe illness, and death. We defined total illness as all acute illness, severe illness, and Q fever–related deaths. Severe illness includes hospitalization during acute infection, chronic illness, Q fever fatigue syndrome (QFS), and death. For pregnant women, the outcome of the unborn child is included in illness estimates: low-birthweight newborns were included in the total illness estimates, and abortions/newborn deaths were included in all 3 outcome categories.

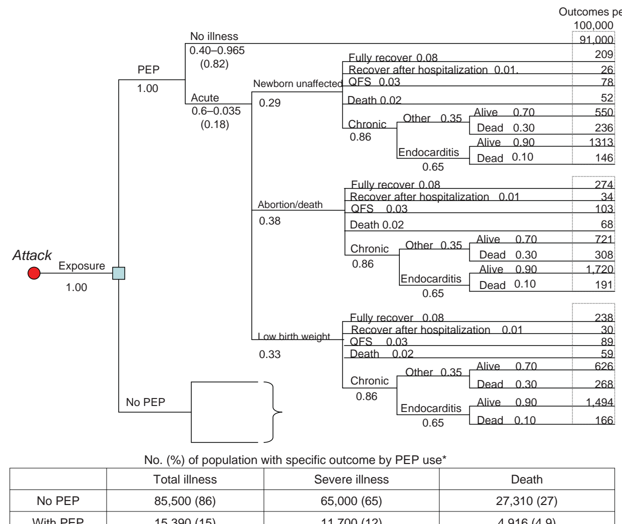
We provide, in Table 1, the values used in the analyses based on information we obtained from an extensive literature review. The probabilities associated with each possible event were multiplied and applied to a population of 100,000 to estimate the number of people who would experience a given outcome with and without PEP (Figures 1–3). Cases averted because of PEP use were calculated (Equation 4 in online Technical Appendix).

PEP-related Adverse Events

The medical literature was reviewed to determine the probabilities of adverse events associated with doxycycline and trimethoprim-sulfamethoxazole. Adverse events were categorized as moderate, severe, or death resulting from prophylactic antibimicrobial drug use. We assumed that a moderate PEP-related adverse event is equivalent to an acute case of Q fever, a severe PEP-related adverse event is equivalent to a severe acute case or chronic case of Q fever, and a death from PEP use is equal to a death from Q fever.

A best estimate and an upper bound estimate for the probabilities of adverse events were selected (Table 2). The best estimates for rates of adverse events from doxycycline are based on estimates cited in a study of anthrax prophylaxis–related adverse events (for both doxycycline and ciprofloxacin) (*33*). The upper bound estimates for doxycycline are arbitrarily defined as 3× the best estimate probabilities. In the case of the upper bound probability of death for doxycycline, because a death rate of 0.0% was stated in the literature (*30,33*), 0.01% was arbitrarily assigned on the basis of the best estimate for severe adverse events (0.01%).

The best estimate for moderate adverse events from TMP-SMX is based on a study that cited 3.9% (7/180) of patients discontinued antimicrobial drug treatment based on adverse events (*34*). Two other studies reported that 11% of patients prematurely discontinued TMP-SMX use based on adverse events (*24,35*). However, these studies likely over



No. (%) of population with specific outcome by PEP use*

	Total illness	Severe illness	Death
No PEP	85,500 (86)	65,000 (65)	27,310 (27)
With PEP	15,390 (15)	11,700 (12)	4,916 (4.9)

Figure 3. Decision tree for 100,000 pregnant women based on an assumption of an aerosolized, point source, overt attack with *Coxiella burnetii* (postexposure prophylaxis [PEP] with 160/800 mg trimethoprim-sulfamethoxazole 2×/d for duration of pregnancy, assuming 82% efficacy and 100% exposure.) PEP-related adverse events are not included in this figure. The “No PEP” segment of the tree contains the same branches and nodes as seen in the “With PEP” section, but uses different probabilities for certain variables. *The outcome of the unborn child is included in pregnant women illness estimates: low birth weight newborns were included in “Total Illness” estimates, and abortions/newborn deaths were included in all 3 outcome categories. QFS, Q fever fatigue syndrome.

Table 1. Input values used in the primary and secondary analyses of PEP efficacy*

Variable	Primary analysis (sensitivity analysis)	Sensitivity analyses		References
		Less virulent	More virulent	
Exposure	(0.10, 0.25, 0.50, 1.00)	NA	NA	NA
Efficacy of doxycycline PEP (8–12 d postexposure)	0.82 (0.82–0.965)	0.965	0.40	(*22,23*)
Efficacy of trimethoprim-sulfamethoxazole PEP (8–12 d postexposure)	0.82 (0.40–0.965)	0.965	0.40	(*21,24,25*)
Asymptomatic infection w/o PEP (all groups)	0.50	0.65	0.40	(*1,3,5,7,8,26*)
Full recovery after acute (gp)	0.74	Residual (0.934)	Residual (0.576)	(*7–9*)
Full recovery after acute illness (hr)	0.28	Residual (0.739)	Residual (0.076)	(*7–9*)
Full recovery after acute illness (pw)	0.08	Residual (0.57)	Residual (0.02)	(*7–9*)
Probability of hospitalization and recovery given acute illness (gp)	0.04	0.01	0.05	(*5,7,27*)
Probability of hospitalization and recovery given acute illness (hr)	0.01	0.01	0.05	(*5,7*)
Probability of hospitalization and recovery given acute illness (pw)	0.01	0.01	0.02	(*5,7*)
Q fever fatigue syndrome (gp)	0.20	0.05	0.30	(*1,10,16,28–30*)
Q fever fatigue syndrome (hr)	0.30	0.05	0.20	(*1,10,16,28–30*)
Q fever fatigue syndrome (pw)	0.03	Residual (0.03)	Residual (0.04)	(*1,10,16,17,28–30*)
Death from acute illness (gp)	0.01	0.001	0.024	(*7,9–11*)
Death from acute illness (hr and pw)	0.02	0.001	0.024	(*7,9–11*)
Chronic disease (gp)	0.01	0.005	0.05	(*8,11,12,26,31*)
Chronic disease (hr)	0.39	0.20	0.65	(*13,14,31*)
Chronic disease (pw)	0.86	0.39	0.90	(*17*)
Endocarditis (all groups)	0.65	0.60	0.90	(*1,7,8*)
Death from endocarditis (all groups)	0.10	0.05	0.60	(*1,3,7,8,11,15,32*)
Death from other chronic diseases (all groups)	0.30	0.05	0.60	(*9*)
Abortion or neonatal death	0.38	0.25	0.56	(*8,14 ,17,21*)
Premature birth/low birth weight baby	0.33	0.25	0.28	(*8,14,17,21*)
Healthy, unaffected baby	0.29	0.50	0.16	(*8,14,17,21*)

*PEP, postexposure prophylaxis; NA, not applicable; gp, general population; hr, high-risk; pw, pregnant women. See online Appendix Table 1 (available from www.cdc.gov/EID/content/14/10/1558-appT1.htm) for a detailed explanation of how the primary input values were selected.

estimate the probability of moderate AEs as some patients may discontinue use after experiencing only mild adverse events. Therefore, the lowest percent cited in the literature (3.9%) was used as the best estimate, and 11% was set as the upper bound estimate. We note that this best estimate may still be an overestimate.

A probability of 0.00037% was selected for the adverse deaths from TMP-SMX use, which is derived from a study that estimated 3.7 deaths/million treatments (*36*). We assumed that most deaths from TMP-SMX treatment are a result of toxic epidermal necrolysis (TEN) (*37*). A severe adverse event probability of 0.00123% is based on the estimate that 30% of TEN cases result in death (*37*).

As stated above, the TMP-SMX upper bound estimate for moderate adverse events was set at 11% (*24,35*). The TMP-SMX upper bound severe AE estimate, 0.0037%, was obtained by multiplying the TMP-SMX best estimate for severe AEs by 3. This was done to remain consistent with the arbitrary selection of an upper bound severe adverse event estimate for doxycycline, which used the same selection technique. Lastly, 0.0011% was used as the upper bound estimate for TMP-SMX-related deaths on the basis of the aforementioned assumption that 30% of severe adverse events (i.e., TEN) result in death (*37*). Because of lack of relevant data, and to avoid underestimating drug-related side effects, we assumed the upper bound estimate of doxycycline-related deaths to be ≈10× greater than that of TMP-SMX (Table 2).

Threshold Point

The threshold point is defined as the probability of exposure to *C. burnetii* where the number of PEP-related adverse events equals the cases averted because of PEP use. The risk for adverse events equals the benefit of PEP use.

Sensitivity Analyses

We conducted initial sensitivity analyses on the efficacy of doxycycline (96.5% and 82%) and TMP-SMX (96.5%, 82%, and 40%). These drug efficacies were chosen on the basis of a review of the literature (Table 1; online Appendix Table 1, available from www.cdc.gov/EID/content/14/10/1558-appT1.htm), but because of lack of evidence of TMP-SMX's efficacy as a prophylaxis for Q fever, we arbitrarily chose a low-range efficacy value (40%). Because of uncertainty in many of the input values for the

Table 2. Probability of adverse events associated with postexposure prophylactic antimicrobial agents

Level of adverse event	Doxycycline*		Trimethoprim-sulfamethoxazole†	
	Best estimate	Upper bound†	Best estimate	Upper bound
Moderate	1.01%	3.03%	3.90%	11%
Severe	0.01%	0.03%	0.00123%	0.00370%‡
Death	0.00%	0.01%	0.00037%	0.00111%

*Recommended for the general population and high-risk populations based on estimated use of 100 mg orally 2×/d for 5 d.
†Recommended for pregnant women based on 160 mg/800 mg orally 2×/d for the duration of the pregnancy.
‡Arbitrary upper-bound, 3× best estimate (see text for further details).

primary analyses (Table 1), we conducted 2 additional scenarios labeled less virulent and more virulent. "Less virulent" and "more virulent" are the terms used to describe the lower and upper bound of the sensitivity analyses. The less virulent values are those that create a best-case scenario for health outcomes, while the more virulent analysis uses the worst-case scenario values.

As appropriate, we reduced (for less virulent) or increased (for more virulent) the input values used in the primary scenario (Table 1; online Appendix Table 1). In many instances, we did not have reliable measures to define less or more virulence and values were assumed as needed. As before, we ran each of the altered virulence scenarios assuming different levels of drug efficacy (doxycycline, 82% and 96.5%; TMP-SMX, 40%, 82%, and 96.5%).

Results

We estimate that without the use of postexposure prophylaxis, exposing a general population of 100,000 to *C. burnetii* would result in 50,000 cases of illness, 13,000 severe cases, and 585 deaths (Figure 1). Figures 2 and 3 provide results for the high-risk population and pregnant women, respectively. If we assume 82% drug efficacy for doxycycline, 9,000 cases of illness, 2,340 severe cases, and 105 deaths would occur within an exposed general population that took PEP. This translates to 41,000 cases of illness, 10,660 severe cases, and 480 deaths averted because of PEP use (82% reduction of cases). In addition, using doxycycline as PEP in a population of 100,000 (using the adverse event best estimates found in Table 2) would result in ≈1,010 moderate adverse events, 10 severe adverse events, and 0 deaths. Therefore, subtracting these adverse events from the total PEP-averted cases show that PEP use in this population would prevent 39,990 cases of total illness, 10,650 severe cases, and 480 deaths. Table 3 displays the total medical cases averted (accounting for PEP-related adverse events) for each group.

Figures 1–3 also include the percentage of each population that would develop illness/death with and without the use of PEP. Of particular importance is the probability of severe cases of illness without PEP use; 13% of the general population, 36% of the high-risk population, and 46% of pregnant women would experience severe illness. Of all cases of illness among the general population, the high-risk population and pregnant women, 26%, 72%, and 92%, respectively, would be severe. Moreover, abortion or newborn death would occur in 19% of exposed pregnant women; 16.5% would give birth to a low-birthweight baby.

The threshold point is defined as the probability of exposure to *C. burnetii* where the risk for adverse events equals the benefit of PEP use. Figure 4 illustrates the general population threshold points (run at 2 different drug efficacy values) for total cases of illness averted for the primary, less, and more virulent scenarios. The x-intercept on these graphs is the probability of exposure to *C. burnetii* at which the total number of cases of illness averted because of PEP use is equal to the number of moderate PEP-related adverse events. Therefore, for any probability of exposure greater than the stated threshold value, PEP would prevent more cases of illness than the number of adverse events PEP would cause. As Figure 4 illustrates, the less virulent or more virulent scenarios affect the estimated number of cases but do not greatly affect the threshold probabilities of exposure. For further analyses, refer to online Appendix Tables 2 (available from www.cdc.gov/EID/content/14/10/1558-appT2.htm), 3 (available from www.cdc.gov/EID/content/14/10/1558-appT3.htm), and 4 (available from www.cdc.gov/EID/content/14/10/1558-appT4.htm) to review univariate sensitivity analyses on various variables used in the risk/benefit scenarios. These tables show which variables have the greatest independent influence on the respective outcomes

Table 3. Total medical cases averted because of postexposure prophylaxis*

Population	No. cases averted
General population	
All cases of illness	39,990
Severe illness	10,650
Deaths	480
High-risk population	
All cases of illness	39,990
Severe illness	29,510
Deaths	3,538
Pregnant women†	
All cases of illness	66,210
Severe illness	53,300
Deaths	22,394

*Accounting for best estimate drug-related adverse events, 82% drug efficacy, and 100% exposure.
†Includes the outcome of the unborn child.

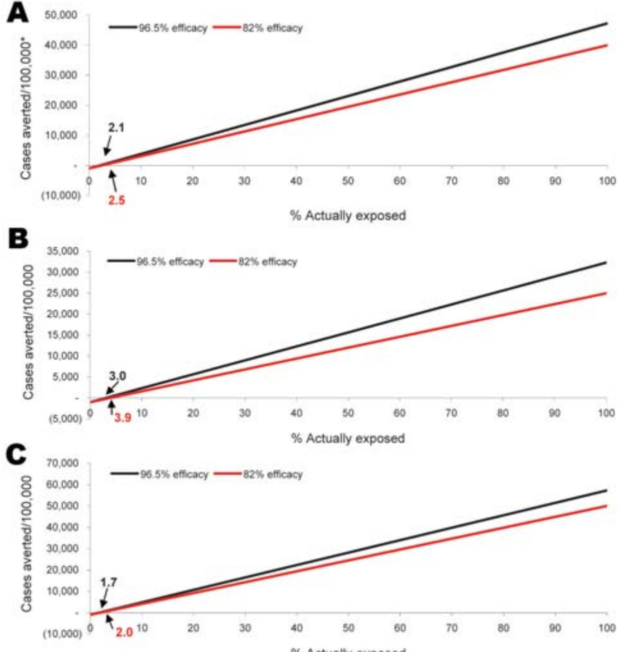


Figure 4. Cases of illness averted in the general population after *Coxiella burnetii* exposure with the use of postexposure prophylaxis while accounting for a 1.0% probability of adverse events, broken down by virulence scenario and drug efficacy. The “best estimate” scenario (primary analysis, A) uses best estimate input values, the “less virulent” scenario (B) uses input values that result in the least harmful outcome, the “more virulent” scenario (C) applies input values that result in the most harmful outcome or worst-case scenario. Drug efficacy refers to the efficacy of doxycycline as a post-exposure prophylaxis against *C. burnetii* infection. Analyses for doxycycline (used by the general and high-risk populations) were run at 2 potential drug efficacies: 96.5% and 82%. The threshold points, the probability of exposure where the risk of adverse events equals the risk of illness, are noted on the graphs.

and how modifications to the input values impacts the estimated number of cases averted.

Table 4 displays all threshold points by group, outcome, drug efficacy, and probability of an adverse event (best or upper bound estimate). Threshold points (when using primary analysis input values) range from 0.0% to 7.4% for the general population and high-risk groups; the threshold points for pregnant women range from 0.001% to 32.2%.

Discussion

Based on this study, we believe many cases of illness and deaths could be prevented with the use of PEP after a deliberate, overt release of *C. burnetii*. Without taking social or political concerns into account, a threshold point can be interpreted as the decision point for PEP use. Any value above the threshold point indicates that the benefits of PEP use outweigh the risks for adverse events, therefore implying PEP should be recommended for any probability of exposure to *C. burnetii* above the stated threshold point. For the general and high-risk population, when doxycycline is used as a postexposure prophylactic antimicrobial drug, due to low rates of AE, the argument to administer PEP in most cases of potential exposure is strong. Even in the worst case scenario (upper bound adverse event estimate), the threshold point for total illness is relatively low at 7.4%.

Use of TMP-SMX for pregnant women also favors PEP use in most scenarios. Due to higher rates of moderate adverse events among TMP-SMX users, the threshold point for total illness is not as low as seen for doxycycline users (the general population and high-risk populations), but TMP-SMX threshold points still exhibit the importance of providing prophylaxis. Although the upper bound AE estimate in conjunction with the lower bound estimate of drug efficacy (40%) indicates 32.2% is the threshold point for total illness, this is the worst case scenario. Both the upper bound estimate for adverse events and the upper bound drug efficacy are considered to be overestimations to the preferred best estimate. The efficacy of TMP-SMX as a form of PEP is likely closer to 82% on the basis of its efficacy as a prophylaxis for several infections such as toxoplasmosis and *Pneumocystis carinii* pneumonia (*25*). Moreover, because Q fever is primarily an incapacitating agent, severe illness is likely a good proxy for the disease’s effects on a population. Therefore, the severe case threshold point (assuming 82% drug efficacy) is low, lying between 0.002% and 0.007% for pregnant women, which provides strong support for PEP use in most cases where exposure is suspected.

On the basis of these analyses, we determined there are 2 variables that most strongly influence the model. First, the efficacy of the drug as prophylaxis for Q fever is 1 of the most important variables in this model. Understandably, if the antimicrobial agent is effective, considerable illness and death will be prevented. Unfortunately, there are limited data on the efficacy of these drugs at preventing Q fever illness. Sensitivity analysis was conducted on this variable to account for this uncertainty; however, based on treatment experiences with these drugs (doxycycline’s efficacy ranges from 82% to 99% for *Chlamydia trachomatis* cervicitis) (*22,23*), we think the best estimates used in this study (82%) are conservatively close to the actual drug efficacies. A second important variable in the model is the probability of PEP-related adverse events. Once again, attempts were made to account for limited data by providing best and upper bound estimates for adverse events.

Although this risk-benefit analysis may be very useful when developing policy and official PEP recommendations, there are limitations to this design, such as some

Table 4. Summary of threshold points by group, drug efficacy, and probability of an adverse event (best estimate/upper bound estimate)

Population	Drug efficacy				
			82%		
	96.50%	40%*	Primary analysis	Less virulent	More virulent
General population					
All cases of illness	2.1/6.3	NA	2.5/7.4	3.88/11.65	1.98/5.94
Severe illness	0.08/0.24	NA	0.09/0.28	0.58/1.75	0.05/0.14
Death	0.00/1.8	NA	0.00/2.1	0.00/28.49	0.00/0.36
High-risk population					
All cases of illness	2.1/6.3	NA	2.5/7.4	3.88/11.65	1.98/5.94
Severe illness	0.03/0.09	NA	0.03/0.10	0.147/0.44	0.021/0.06
Death	0.00/0.24	NA	0.00/0.28	0.00/3.5	0.00/0.05
Pregnant women					
All cases of illness	4.7/13.3	11.4/32.2	5.6/15.7	9.99/28.2	4.16/11.7
Severe illness	0.002/0.006	0.005/0.014	0.002/0.007	0.0069/0.021	0.0016/0.0047
Death	0.001/0.004	0.003/0.010	0.002/0.005	0.0053/0.0156	0.0006/0.0019

*Doxycycline was not evaluated at 40% drug efficacy. NA, not available.

of the data on which this analysis is based. Particularly, the recommendation that PEP should be administered 8–12 days postexposure is based on a single study conducted on only 5 persons and 1 type of antimicrobial drug (oxytetracycline). We acknowledge that these are limited data, but administration 8–12 days postexposure still remains the official recommendation of the US Army Medical Research Institute of Infectious Diseases. As a result, we remain consistent with current recommendations, but we are open to alternative options if more evidence becomes available. Conducting further animal studies would help to clarify the optimal time and duration of drug administration and the ideal antimicrobial drug. Newer drugs are now available and these may be more effective at preventing illness.

Another limiting variable was the death rate from acute Q fever infection among non-PEP users. Our study assumed that all persons in whom acute or chronic illness develops are assumed to receive appropriate treatment and care for the duration of illness once a diagnosis of Q fever has been made. Although no estimates are available in the literature for the death rate among treated persons (only untreated), we chose to use the death rate for untreated persons (1%). However, because of the uncertainty of this value, sensitivity analyses were conducted to assess variable effect on the number of severe cases of illness averted. Online Appendix Table 4 shows how the change in input values for both the PEP and no PEP groups does not greatly impact the total number of severe cases of illness averted within the general population.

Also, as mentioned before, this risk-benefit analysis is based on several assumptions, such as an overt attack, 100% exposure, and 100% compliance of the entire study population. These assumptions simplify the situation and create a more quantifiable, but more unnatural, scenario. Although an overt attack is less likely to occur, future models can adjust this assumption to account for a delay in diagnosis or outbreak detection. Prophylactic efficacy and ultimately the number of illnesses/deaths could vary depending on such factors as compliance and the number of organisms to which the person was exposed. Another limitation to acknowledge is that *C. burnetii* is very resilient in the environment and exposure a long time after the initial dispersal could be an issue. Our model has only accounted for a single-exposure event, but future models should address this point.

Several issues will also be important when considering PEP recommendations. First, when selecting a representative threshold value for each risk group, social and political concerns must be acknowledged and considered. If a threshold value is 2.5%, it may be more realistic for decision makers to instruct all persons with any probability of exposure to take PEP rather than use valuable time and resources to determine a person's numerical probability of exposure. In addition, before providing a specific numeric threshold value in the guidelines, knowing how to measure that probability of exposure is important. For example, if 7% is provided as a threshold, there must be a mechanism for differentiating between 6% and 8% or 5% and 15%. These threshold points and PEP recommendations must be useful and realistic. Some research has been conducted to evaluate how to determine likely concentrations of a bioterrorism agent and a person's level of exposure by using computer modeling and simulation (*38*). Further studies on the assessment of exposure would be beneficial; modeling Q fever exposure would be especially critical given *C. burnetii*'s low infectious dose and high environmental stability (*9*).

Also, this study does not directly address children <8 years of age. In general, this population is not at higher risk for illness/death from Q fever infection than the general population. However, risk-benefit analyses for children should be conducted to provide guidance on PEP recommendations for this age group. Lastly, this analysis was

conducted on the basis of the most frequently suggested prophylaxis regimens. However, other antimicrobial drugs should be evaluated with risk-benefit analytic methods.

Cost was not considered in this risk-benefit analysis. Further studies are warranted to expand and support various aspects of this analysis, including estimating the cost associated with the use of PEP after a deliberate release of *C. burnetii*.

This study illustrates the importance and benefit of postexposure prophylaxis in a mass-exposure scenario and also weighs the risk for prophylaxis-related adverse events. Early identification of persons at increased risk for Q fever illness (pregnant women and high-risk populations) would be crucial in providing proper PEP and, in turn, preventing illness/death in these groups. Based on the study assumptions of exposure and compliance, PEP may be warranted and is likely to be effective at averting cases of illness and deaths in all 3 population groups when the probability of exposure to *C. burnetii* is above the population-specific threshold point.

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etymologia

Coxiella [kok″ se-el′ ə] *burnetii*

Etiologic agent of Q fever, named after American bacteriologist Herald Rea Cox and Australian physician Frank MacFarlane Burnet, who both independently isolated the bacterium in the 1930s. *C. burnetii* belongs in the family *Coxiellaceae*, which consists of gram-negative rods without flagella or a capsule. The bacteria occur in ticks and various vertebrates, including humans.

Source: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; American Veterinary Medical Association. Q fever backgrounder. Available from http://www.avma.org/public_health/biosecurity/qfever_bgnd.asp

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Prophylaxis after Exposure to *Coxiella burnetii*

Cryptosporidium Species and Subtypes and Clinical Manifestations in Children, Peru

Vitaliano A. Cama, Caryn Bern, Jacqueline Roberts, Lilia Cabrera, Charles R. Sterling, Ynes Ortega, Robert H. Gilman, and Lihua Xiao

To determine whether clinical manifestations are associated with genotypes or subtypes of *Cryptosporidium* spp., we studied a 4-year longitudinal birth cohort of 533 children in Peru. A total of 156 infection episodes were found in 109 children. Data from first infections showed that *C. hominis* was associated with diarrhea, nausea, vomiting, general malaise, and increased oocyst shedding intensity and duration. In contrast, *C. parvum*, *C. meleagridis*, *C. canis*, and *C. felis* were associated with diarrhea only. *C. hominis* subtype families were identified (Ia, Ib, Id, and Ie); all were associated with diarrhea. Ib was also associated with nausea, vomiting, and general malaise. All *C. parvum* specimens belonged to subtype family IIc. Analysis of risk factors did not show associations with specific *Cryptosporidium* spp. genotypes or subtypes. These findings strongly suggest that *Cryptosporidium* spp. and subtypes are linked to different clinical manifestations in children.

Cryptosporidiosis is often observed as a pediatric disease in areas where *Cryptosporidium* spp. are endemic. Children <2 years of age are frequently infected in theses areas in community (*1–4*) and hospital (*5*) settings. The spectrum of symptoms is diverse, ranging from acute diarrhea, severe chronic diarrhea (*6*), or vomiting to asymptomatic infections (*2,3*). In community-based studies in Peru, ≈30% of immunocompetent children with cryptosporidiosis reported diarrhea (*2,7*). In AIDS patients, the diversity of symptoms has been linked to immune status; severe chronic diarrhea affects patients whose CD4+ counts are <200 cells/mm^3 (*8*). A recent study in HIV-infected patients in Peru showed that only 38% with *Cryptosporidium* infections had diarrhea (*9*), although 64% of participants had CD4+ counts <200 cells/mm^3. However, the cause for these variations is not clearly understood.

The use of molecular tools in epidemiologic investigations has provided new insights into the diversity of *Cryptosporidium* spp. infecting humans and animals (*10*). There are at least 16 established *Cryptosporidium* spp. and >40 unnamed genotypes that are potentially different species. At least 8 of them have been reported in humans: *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, and *C. suis*, and the *Cryptosporidium* cervine genotype. Molecular characterization of the 60-kDa glycoprotein (GP60) gene of *C. hominis* and *C. parvum* has enabled further division into subtype families and subtypes (*11*).

Humans are most frequently infected with *C. hominis* and *C. parvum* (*7,11,12*); recent reports indicate possible associations between these 2 organisms and different clinical manifestations. In Brazil, children infected with *C. hominis* had increased parasite shedding, more frequent presence of fecal lactoferrin, and delayed growth when compared with those infected with *C. parvum* (*13*). In a study of sporadic cryptosporidiosis in the United Kingdom, illness was more severe in persons infected with *C. hominis* than in those infected with *C. parvum* (*14,15*). A recent study reported different clinical manifestations among *Cryptosporidium* spp. in HIV-positive persons, and *C. hominis* was linked to more severe symptoms. The high virulence of *C. hominis* was evident within its subtype family Id, while absent in subtype families Ia and Ie (*16*).

In this study, we analyzed the diversity of *Cryptosporidium* at the species, subtype family, and subtype levels in

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children living in an area with endemic cryptosporidiosis. We also analyzed the association between clinical manifestations and infections with specific *Cryptosporidium* spp. and *C. hominis* subtype families.

Methods

Study Design

Specimens and data were obtained from a longitudinal birth cohort study of diarrheal diseases conducted during 1995–1998 in Pampas de San Juan de Miraflores, Lima, Peru. This community was initially settled in the 1980s by immigrants from rural areas. It is located in the outskirts of Lima and had at the time of the study ≈40,000 inhabitants. In this community, the prevalence of HIV infection was <1% (*2,7*). The study protocol was reviewed and approved by the institutional review boards of Johns Hopkins University and the Centers for Disease Control and Prevention. All participants provided informed consent before participation in the study.

Microscopy

The study participants were asked to provide weekly fecal specimens for microscopic detection of ova and parasites, including *Cryptosporidium* spp. Stool specimens were washed and concentrated by using the modified Ritchie formalin-ether method and examined for *Cryptosporidium* spp. oocysts by microscopy of smears stained with a modified acid-fast stain. Intensity of *Cryptosporidium* spp. oocyst shedding in stools was determined by counting the number of oocysts per 50 μL of concentrated sample. We used a 0 to 3+ scoring system in which 0, negative; 1+, 1–50 oocysts; 2+, 51–150 oocysts; and 3+, >150 oocysts.

Genotyping and Subtyping

Cryptosporidium spp. were identified by using a small subunit rRNA-based PCR–restriction fragment length polymorphism genotyping tool (*7,12,17*). Subtyping of *C. hominis* and *C. parvum* was based on sequence analysis of GP60 genes (*18*). Each specimen was analyzed by either method at least twice. Subtype families within *C. hominis* and *C. parvum* were determined on the basis of sequence differences in the nonrepeat region of the gene. Within each subtype family, subtypes differed from each other, mostly in the number of serine-coding trinucleotide repeats (TCA, TCG, or TCT microsatellite) located in the 5′ region of the gene. The previously established nomenclature system was used to differentiate subtypes within each subtype family (*11,16,17*). For *C. parvum* subtype family IIc, the original GP60 sequence for *C. parvum* subtype family IIc (GenBank accession no. AF164491) was assigned as IIcA5G3a. Subtypes that diverged from this sequence were assigned subsequent alphabetical extensions.

Associated Clinical Manifestations and Risk Factors

Daily information on clinical manifestations was gathered by using structured questionnaires. These data were collected by study personnel during interviews of adult caregivers of the participants. Data included relevant gastrointestinal symptoms such as abdominal pain, fever, general malaise, nausea, vomiting, number and consistency of bowel movements, and blood in stools.

Study of potential risk factors for infections was based on sanitation and socioeconomic data obtained at study enrollment. These factors included hygiene parameters (water piped inside the house and presence of flush toilets), presence of animals (dogs, chicken, ducks, guinea pigs, rabbits, parrots, and sheep), house infrastructure (sturdy walls and roof), and indirect economic indicators (house infrastructure and possession of electronic appliances).

Definitions

For the epidemiologic and statistical analyses, we included data from eligible children who had ≥6 months of participation in the study and <20% noncompliance of study procedures. For the epidemiologic analyses we used the following definitions.

Duration of an infection episode was defined as an episode that started on the first date that *Cryptosporidium* spp. oocysts were microscopically detected in stools and ended on the date of the last positive stool that was followed by at least 3 weekly specimens that were microscopically negative. The length of the infection episode was the number of days between the start and end dates.

An episode of diarrhea was defined as a child having ≥3 liquid or semiliquid bowel movements on any day and the mother's assessment that the child had diarrhea. Diarrhea was considered associated with an episode if it occurred within 7 days of a positive result for *Cryptosporidium* spp.

Statistical Analysis

Statistical analyses included data from participants infected with 1 species of *Cryptosporidium* and compared children with a specific *Cryptosporidium* sp. or *C. hominis* subtype family with all other participants not infected with that species or subtype family. Subtype families were compared because of the extensive sequence polymorphism in the nonrepeat regions of GP60, and subtypes within families primarily differed from each other in the length of the serine stretch at the beginning of the protein. Data from the few children infected with >1 species or subtype determinations that were conflicting with genotype categorizations were excluded from that particular comparison. Because all *C. parvum* in this population belonged to 1 subtype family, results were presented at the species level. Few participants were infected with *C. canis* and *C. felis* and these species

are genetically divergent from *C. hominis*, *C. parvum*, and *C. meleagridis*. Therefore, the data for these persons were pooled.

Poisson regression was used to compare incidence rates of gastrointestinal symptoms (dependent variables) and infections with *Cryptosporidium* spp. or subtype families (independent variables) detected in each infection episode. This model was used to incorporate individual incidence rates of infections and the duration that each person participated in the study. These regression analyses were conducted by using SAS Proc Genmod (SAS Institute, Cary, NC, USA) for linear models. The generalized estimating equations procedure was implemented to adjust for correlation among multiple infections for the same child. Statistical significance for a priori tests was set at $\alpha = 0.05$. Whenever multiple subtypes were compared, a separate Bonferroni adjustment was used to maintain an overall experiment-wide α of 0.05.

The χ^2 or Fisher exact tests were used to analyze any association between *Cryptosporidium* spp. or subtypes and animal contacts or socioeconomic risk factors. Pooled *t* test was used to investigate the differences in age at first infection episode among *Cryptosporidium* spp. and subtype families. All statistical analyses were performed by using SAS version 9.1 (SAS Institute).

Results

A total of 533 children were enrolled, and their median age at enrollment was 14 days. They contributed 44,042 stool specimens for detection of enteric parasites and 324,067 child-days of clinical manifestation surveillance.

Prevalence of Cryptosporidiosis

Data from 368 participants who met the evaluable criteria were included in the epidemiologic analyses. Cryptosporidiosis was detected by microscopy for 109 participants, for a total of 156 infection episodes. Among them, 71 children had 1 infection, 30 had 2 infections, 7 had 3 infections, and 1 had 4 infections.

Cryptosporidium spp. Genotypes and Subtypes

Genotype data for *Cryptosporidium* spp. were obtained from 127 (81%) of 156 infection episodes. Among those genotyped, *C. hominis* (70%) was the species most frequently detected, followed by *C. parvum* (13%) and C. *meleagridis* (8%). In contrast, *C. canis* and *C. felis* were detected in 2% and 5% of cases, respectively (Table 1). Among 106 infection episodes with either *C. hominis* (89) or *C. parvum* (17), subtype analysis was successfully accomplished for 78 of 89 infections with *C. hominis* and 14 of 17 infections with *C. parvum*. Four subtype families were identified within *C. hominis*: Ia, Ib, Id, and Ie, the least frequent was Id. All infections with *C. parvum* belonged to subtype family IIc. Novel subtype sequences were deposited in GenBank under accession nos. EU095258–EU095267 (Table 2).

Table 1. Frequency of infections with *Cryptosporidium* spp. in 533 children, Peru

Species	No. (%) infection episodes	
	First	Overall
C. hominis	61 (64.9)	89 (70.1)
C. parvum	15 (16.0)	17 (13.4)
C. meleagridis	9 (9.6)	10 (7.9)
C. canis	2 (2.1)	2 (1.6)
C. felis	4 (4.3)	6 (4.7)
C. hominis and *C. parvum*	2 (2.1)	2 (1.6)
C. canis and *C. meleagridis*	1 (1.1)	1 (0.8)
No. genotyped	94	127
Total episodes	109	156

Several subtypes were found within subtype families Ia and Id of *C. hominis* and IIc of *C. parvum*. Subtype family Ia was the most diverse with 6 subtypes, followed by subtype families Id and IIc, each with 3 subtypes. In contrast, subtype families Ib and Ie each had only 1 subtype: IbA10G2 was the only subtype in subtype family Ib and IeA11G3T3 was the only subtype in subtype family Ie (Table 2).

Cryptosporidium spp. and Oocyst Shedding

The mean age for first infections was 1.6 years of age (median 1.4 years, range 0.2–4.7 years). Infections with *C. parvum* occurred at a younger age than those with other genotypes, and infections with *C. canis* or *C. felis* occurred in older children. However, these differences were not statistically significant after the Bonferroni correction (Table 3).

The mean duration of the first infection episode was 8.1 days (median 5.5 days, range 1–40 days). Infections with *C. hominis* (mean 10.3 days) lasted longer than infections with other species of *Cryptosporidium* (mean 5.8 days; $p = 0.001$). The length of the infection episodes among children infected with different subtype families of *C. hominis* was not significantly different (9.3, 13.1, 7.7, and 12.8 days for Ia, Ib, Id, and Ie, respectively).

Similar patterns were observed for intensity of parasite excretion. Children infected with *C. hominis* had higher parasite excretion scores (mean 1.93) than those infected with other species of *Cryptosporidium* (mean 1.42; $p = 0.021$). Among children infected with different subtype families of *C. hominis*, the intensity of parasite shedding was similar.

Sequential *Cryptosporidium* spp. Infections

Among children with complete genotyping data, sequential infections were detected in 17 children: 15 had 2 episodes of *Cryptosporidium* spp. infection and 2 had 3 episodes (total of 19 reinfection events). The median in-

Table 2. Distribution of subtype families and subtypes of *Cryptosporidium hominis* and *C. parvum* in 533 children, Peru

Species	Subtype families	No. episodes (%)		Subtype: no. (%) within subtype family	GenBank accession no.
		At first infection	All		
C. hominis	Ia	15 (24.6)	21 (26.9)	IaA11R4: 3 (14)	EU095258*
				IaA12R4: 7 (33)	EU095259*
				IaA13R4: 1 (5)	EU095260*
				IaA13R7: 1 (5)	EU095261*
				IaA14R6: 5 (24)	EU095262*
				IaA15R3: 3 (14)	EU095263*
	Ib	16 (26.2)	23 (29.5)	IbA10G2: 23 (100)	AY262031
	Id	7 (11.5)	12 (15.4)	IdA10: 9 (75)	EU095264*
				IdA15: 1 (8)	DQ280498
				IdA20: 2 (16)	EU095265*
	Ie	15 (24.6)	19 (24.4)	IeA11G3T3:19 (100)	DQ665689
	Ib + Ie	1 (1.6)	1 (1.3)	1 (1.3)	
	Ib + id	1 (1.6)	1 (1.3)	1 (1.3)	
	Id + ie	1 (1.6)	1 (1.3)	1 (1.3)	
C. hominis and *C. parvum*	Id + IIc		1		
C. parvum	IIc	14 (100)	14 (100)	IIcA5G3a: 12 (86)	AY738195
				IIcA5G3b: 1 (7)	EU095266*
				IIcA5G3c: 1 (7)	EU095267*

*From this study.

terval between infections was 10 months (range 2.1–26 months). The same *Cryptosporidium* sp. was detected in 6 of 15 children with 2 episodes and 1 of 2 children with 3 infections, all involving *C. hominis* (Table 4). When analysis of reinfections included *C. hominis* subtype family data, only 2 sequential infections occurred with the same subtype family: child 5395 had *C. hominis* subtype family Id in the first and second infections, and child 5076 had *C. hominis* subtype family Ie in the second and third episodes of cryptosporidiosis.

Cryptosporidium spp. and Subtypes and Associated Clinical Manifestations

Distribution of species and subtype families at first infection among 109 *Cryptosporidium* spp.–infected children was similar to the distribution in all infection episodes. A second model analyzed the data from all infection episodes (Table 5).

On the basis of microscopy results, 36% of infected children had diarrhea, 28.4% had general malaise, 16.5% had abdominal pain, 15.7% had vomiting, and 7.9% had nausea. None of the study participants reported fever or blood in stools. Overall, 44.1% reported ≥1 of the manifestations assessed in the study.

Associated clinical manifestations at first infection varied among different *Cryptosporidium* spp. First infections with *C. hominis* were associated with nausea, vomiting, general malaise, and diarrhea (Table 5). In contrast, infections with other species were associated with diarrhea only.

Patterns of clinical manifestations also varied among *C. hominis* subtype families. Infections with subtype family Ib were associated with nausea, vomiting, general malaise, and diarrhea. Infections with other subtype families (Ia, Id, and Ie) were generally associated with diarrhea only. A similar trend was also seen in the cumulative analysis of all infection episodes at the species and subtype family levels. A possible exception was *C. hominis* subtype family Ia, which showed an association with nausea and vomiting at first infections but did not show such

Table 3. Age at first infection by *Cryptosporidium* spp. and subtype family in 533 children, Peru

Species or subtype family	No. episodes	Age, y, mean (range)	p value
C. hominis	61*	1.93 (0.19–9.51)	0.026†
Subtype family Ia	15	2.13 (0.67–8.05)	0.113
Subtype family Ib	16	1.38 (0.60–2.82)	0.176
Subtype family Id	7	1.41 (0.19–3.34)	0.645
Subtype family Ie	15	1.81 (0.25–9.51)	0.723
C. parvum	15	1.22 (0.44–2.49)	0.034†
C. meleagridis	9	1.43 (0.78–2.75)	0.615
C. canis or *C. felis*‡	6	2.26 (0.68–3.74)	0.039†
Mixed infections	2	1.62 (1.44–1.79)	Not done

*Eight *C. hominis* infections did not have subtype family data.
†Not significant after Bonferroni adjusted $\alpha = 0.05/5 = 0.01$.
‡Includes 1 mixed infection with *C. meleagridis* and *C. canis*.

Table 4. *Cryptosporidium* spp. and subtype families of *C. hominis* detected in reinfection events in 533 children, Peru

Event	Infection		
	First	Second	Third
5444	*C. parvum* (IIc)	*C. hominis* (Id and Ie)	*C. hominis* (Ib)
5076	*C. hominis* (Id)	*C. hominis* (Ie)	*C. hominis* (Ie)*
E392	*C. hominis* (Ib)	*C. hominis* (Ie)	
K283	*C. hominis* (Ib)	*C. hominis* (Id)	
5395	*C. hominis* (Id)	*C. hominis* (Id)*	
5125	*C. hominis*	*C. hominis* (Ia)	
D037	*C. hominis*	*C. hominis* (Ia)	
5492	*C. hominis*	*C. hominis* (Id)	
5471	*C. hominis* (Ib)	*C. parvum*	
5399	*C. hominis* (Ie)	*C. felis*	
5370	*C. parvum*	*C. hominis* (Id)	
5266	*C. meleagridis*	*C. hominis* (Ib)	
H131	*C. meleagridis*	*C. hominis* (Ia)	
5082	*C. meleagridis*	*C. hominis*	
5300	*C. felis*	*C. hominis*	
5085	*C. canis*	*C. hominis*	
5049	*C. hominis* and *C. parvum*	*C. felis*	

*Reinfections with the same subtype family.

an association in the cumulative analysis of all infection episodes (Table 5).

Discussion

Rates of clinical manifestations in our study were lower than rates reported for a birth cohort in Brazil, where 81% of 42 participants infected with *C. hominis* or *C. parvum* had diarrhea (*13*). This difference can be attributed to differences in study designs. Our study analyzed weekly stool samples for the presence of *Cryptosporidium* spp. and other parasites in a cohort of healthy children. In contrast, the cohort study in Brazil was designed to identify causes of diarrhea, and the specimens were collected within 2 weeks of clinical identification of diarrhea.

C. hominis was the predominant species in this community-based longitudinal study, followed by *C. parvum* (*7*). This predominance of *C. hominis* has been observed in persons in other developing countries, such as pediatric populations from Malawi (*19*), Kenya (*20*), India (*21*), Haiti (*22*), and Brazil (*13*), children and elderly persons from South Africa (*23*), and hospitalized HIV-infected children from South Africa and Uganda (*24,25*). As reported in previous studies (*21,24,26,27*), we also detected few concurrent infections with multiple *Cryptosporidium* spp. or *C. hominis* subtype families.

We observed a comparatively large proportion of participants infected with *C. meleagridis*, a finding that was also reported at a high frequency in HIV-infected adults in Lima, Peru (*12,16*). This species has been rarely reported for studies from other locations such as Portugal (*28*), India (*21,26,29*), Taiwan (*30*), or Iran (*31*) that included either children or adults with or without HIV infections. It should be noted that the diversity of *Cryptosporidium* spp. is also affected by the methods used. We used a genotyping tool proven to distinguish several dozen species and genotypes. However, methods based on genes coding for a 70-kDa heat-shock protein (*32*), *Cryptosporidium* spp. oocyst wall protein (*33*), or a smaller fragment of the small subunit rRNA gene (*34*) discriminate fewer *Cryptosporidium* spp. and genotypes.

Overall, distribution of species and *C. hominis* subtype families in our study was similar to that found in an HIV study in Lima, Peru (*12,16*). These 2 studies were conducted in the same area but in different study populations. In both studies, all *C. parvum* specimens belonged to subtype family IIc, which is considered anthroponotic in origin (*17*). The normally zoonotic subtype family IIa was not seen in our study population. This finding is also supported by our risk factor data, which showed the lack of bovines in the study households and the absence of cattle farms in or near the community of Pampas de San Juan. The similarity of the species and subtype distribution in both studies is highly suggestive that the prevalence of *Cryptosporidium* spp. and subtypes in a specific location is independent of the immune status of the study population.

The role of parasite genetics in clinical manifestations of cryptosporidiosis is not clear. Studies of human volunteers showed that exposure provided some degree of protection against infection and illness; the infection rates and frequencies of infection-associated clinical manifestations were lower for subsequent infections (*35*). Thus, clinical manifestations caused by parasite differences would be better observed in primary infections. Our longitudinal birth cohort study enrolled children at an early age (median 14 days), which enabled us to study genotypes and subtypes present at first infections and their associations with different clinical manifestations.

First infections with all species and *C. hominis* subtype families were associated with diarrhea. However, only *C. hominis* subtype family Ib was also associated with nausea, vomiting, and general malaise, but *C. hominis* subtype families Ia, Id, and Ie, and other *Cryptosporidium* spp. were not. Previously, other studies had suggested that *C. hominis* might be more pathogenic than other species or might induce different clinical manifestations (*13,15,21*). Our results indicate that within *C. hominis*, subtype family Ib may be more pathogenic than Ia, Id, and Ie. Subtype family Ib of *C. hominis* is the most frequently detected *Cryptosporidium* spp. in waterborne outbreaks of cryptosporidiosis in industrialized nations (*36*).

A previous study of cryptosporidiosis in HIV-infected persons in Peru showed that infections with different species or subtype families were associated with different clin-

Table 5. Associations between infections with *Cryptosporidium* spp. or *C. hominis* subtype families and clinical manifestations expressed as incidence rate ratios in 533 children, Peru*

Clinical manifestation	First infection		All infections	
	IRR	p value	IRR	p value
Children infected with *C. hominis* vs. those with cryptosporidiosis but not infected with *C. hominis*				
Nausea	5.469	<0.001†	3.531	0.037‡
Vomiting	2.252	<0.001†	2.359	0.023‡
General malaise	2.523	<0.001†	2.071	0.035‡
Diarrhea	3.690	<0.001†	4.886	<0.001†
Children infected with *C. parvum* vs. those with cryptosporidiosis but not infected with *C. parvum*				
Diarrhea	3.249	<0.001†	4.562	<0.01†
Children infected with *C. meleagridis* vs. those with cryptosporidiosis but not infected with *C. meleagridis*				
Diarrhea	2.484	0.006†	7.684	<0.001†
Children infected with *C. canis* or *C. felis* vs. those with cryptosporidiosis but not infected with these species				
Diarrhea	3.122	0.002†	1.528	0.662
Children infected with *C. hominis* subtype family Ia vs. those not infected with that subtype family				
Nausea	5.020	<0.001†	3.203	0.183
Vomiting	2.280	0.013‡	2.100	0.141
Diarrhea	2.442	<0.001†	3.061	0.002†
Children infected with *C. hominis* subtype family Ib vs. those not infected with that subtype family				
Nausea	12.516	<0.001†	8.402	0.006†
Vomiting	4.752	<0.001†	4.868	0.004†
General malaise	4.939	<0.001†	4.139	0.006†
Diarrhea	5.510	<0.001†	6.506	<0.001†
Children infected with *C. hominis* subtype family Id vs. those not infected with that subtype family				
Diarrhea	2.999	<0.001†	3.171	0.022‡
Children infected with *C. hominis* subtype family Ie vs. those not infected with that subtype family				
General malaise	1.830	0.010‡	1.613	0.333
Diarrhea	3.117	<0.001†	4.160	0.001†

*Determined by Poisson regression analyses. IRR, incidence rate ratio.
†Statistically significant at Bonferroni corrected $\alpha = 0.01$.
‡Statistically significant at $p<0.05$.

ical manifestations. Patients infected with subtype families Ib and Id of *C. hominis*, *C. parvum*, or *C. canis*/*C. felis* were more likely to have chronic diarrhea, and patients infected with *C. parvum* were more likely to have infection-associated vomiting (*16*). Overall, subtype family Id was the most virulent in the HIV study and was strongly associated with diarrhea in general and chronic diarrhea in particular. Subtype family Ib was also marginally associated with diarrhea and vomiting but not with chronic diarrhea. In this study, however, Id was only associated with diarrhea. This difference may be caused by the fact that chronic cryptosporidiosis, the life-threatening manifestation of the disease in AIDS patients, was never detected in this study of pediatric patients, and few children in this study were infected with subtype family Id, which might have prevented us from assessing its clinical manifestations fully. Nevertheless, our study corroborated the previous observation of defined patterns of clinical manifestations associated with different *Cryptosporidium* spp. and *C. hominis* subtype families.

We also conducted a risk factor analysis for predictors of infection, including age at first infection, in which we did not identify statistically significant associations between any *Cryptosporidium* spp. or subtype families and any of the variables analyzed, although they covered basic aspects of sanitation and zoonotic, foodborne, and waterborne transmission. One possible explanation is that our questionnaires did not obtain data on factors that were relevant. However, the same questionnaire successfully identified infection risk factors for other organisms in the same community (*2*). A more likely explanation is that because most *Cryptosporidium* spp. in this study were anthroponotic in origin, children may be constantly exposed to these ubiquitous parasites through different transmission routes. Therefore, single exposure variables were not identified as risk factors. This constant exposure may also fit the age distribution pattern of cryptosporidiosis in the community, in which most cases are found in children <2 years of age, occasionally found in older children, and almost never found in immunocompetent adults. This finding is in contrast to transmission of *Cryptosporidium* spp. in industrialized nations, where infections have been frequently associated with waterborne transmission from either drinking water (*37*) or recreational water (*38*).

In conclusion, clinical manifestations of cryptosporidiosis in healthy populations in disease-endemic areas are likely diverse, and the spectrum of these clinical manifestations can be attributed in part to the different species of *Cryptosporidium* and subtype families of *C. hominis*.

Although further laboratory and longitudinal cohort studies in other disease-endemic areas are needed to validate our observations, these results demonstrate that parasite genetics may play an important role in the clinical manifestations of human cryptosporidiosis. Future studies should be conducted in different geographic settings; they should overcome some potential limitations of this study, such as lack of data on other gastrointestinal pathogens, which might have confounded the clinical findings, and small sample sizes, which had limited the power of the statistical analyses.

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Endemic and Epidemic Lineages of *Escherichia coli* that Cause Urinary Tract Infections

Amee R. Manges, Helen Tabor, Patricia Tellis, Caroline Vincent, and Pierre-Paul Tellier

Women with urinary tract infections (UTIs) in California, USA (1999–2001), were infected with closely related or indistinguishable strains of *Escherichia coli* (clonal groups), which suggests point source dissemination. We compared strains of UTI-causing *E. coli* in California with strains causing such infections in Montréal, Québec, Canada. Urine specimens from women with community-acquired UTIs in Montréal (2006) were cultured for *E. coli*. Isolates that caused 256 consecutive episodes of UTI were characterized by antimicrobial drug susceptibility profile, enterobacterial repetitive intergenic consensus 2 PCR, serotyping, *Xba*I and *Not*I pulsed-field gel electrophoresis, multilocus sequence typing, and phylogenetic typing. We confirmed the presence of drug-resistant, genetically related, and temporally clustered *E. coli* clonal groups that caused community-acquired UTIs in unrelated women in 2 locations and 2 different times. Two clonal groups were identified in both locations. Epidemic transmission followed by endemic transmission of UTI-causing clonal groups may explain these clusters of UTI cases.

Community-acquired extraintestinal infections with *Escherichia coli* range in frequency from 6 to 8 million cases of uncomplicated cystitis per year to 127,500 cases of sepsis per year in the United States (*1*). Urinary tract infections (UTIs) caused by *E. coli* are one of the most common extraintestinal infections in women and, because of their high incidence, are the focus of most epidemiologic studies. The source of *E. coli* for these infections is a person's intestinal tract; however, how these *E. coli* are acquired by the gut is unclear. Risk factors that lead to intestinal colonization with extraintestinal *E. coli* differ from factors associated with development of infection.

Young, otherwise healthy, sexually active women have the highest risk for community-acquired UTIs. The main risk factors for UTI are recent and frequent sexual intercourse, contraceptive use, and a history of UTIs (*2,3*). Treatment for UTIs usually involves a short course of an antimicrobial drug, such as trimethoprim-sulfamethoxazole (TMP-SMZ). Over the past decade, the prevalence of drug resistance in *E. coli* has increased dramatically, complicating management of these infections. Across the United States and Canada, urinary tract isolates of *E. coli* from outpatient clinics showed increased resistance to TMP-SMZ and ampicillin (*4*). A more serious concern has been the gradual increase in fluoroquinolone (e.g., ciprofloxacin) resistance among UTI isolates (*5*).

There is increasing evidence that the *E. coli* that cause UTIs and other extraintestinal infections may be responsible for community-wide epidemics. In 1986–1987, *E. coli* O15:K52:H1 caused an outbreak of community-acquired UTIs and septicemia in South London, England (*6*). The distinctive drug resistance profile of this clonal group contributed to its recognition in London and other areas of Europe and the United States (*7,8*). Other outbreaks of UTI caused by *E. coli* have been described and include a cluster of UTI cases in Copenhagen, Denmark, caused by *E. coli* O78:H10 and a larger outbreak in Calgary, Alberta, Canada, caused by extended-spectrum β-lactamase (ESBL)–producing *E. coli* (*9,10*).

In 2001, we reported that a multidrug-resistant *E. coli* clonal group designated clonal group A (CgA), defined by an enterobacterial repetitive intergenic consensus 2 (ERIC2) PCR and characterized by O11, O77, O17, and O73:K52:H18 serotypes, caused 11% of all *E. coli* UTIs and 49% of all TMP-SMZ–resistant *E. coli* UTIs in 1

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California, USA, community over a 4-month period (*11*). Members of this clonal group were responsible for drug-resistant UTIs in university communities in Michigan and Minnesota and a community in Colorado (*12*), and for pyelonephritis in several states (*13*). We also identified additional clonal groups in a second cross-sectional study in Berkeley, California (*14*).

Identification of outbreak strains of *E. coli* that cause extraintestinal infections suggests that point sources, possibly contaminated food, may be responsible for local spread of genetically related *E. coli* strains in the United Kingdom. Recent work in the United Kingdom has focused on a possible link between the increase in ESBL-producing *E. coli* and food animal production. An estimated 30,000 cases of human infection with ESBL-producing *E. coli* occur each year in the United Kingdom, and studies have found epidemic strains of ESBL-producing *E. coli* in the United Kingdom and throughout the world (*15–17*). The Health Protection Agency has suggested that imported chicken may be a route for introduction of ESBL-producing *E. coli* into the United Kingdom. Recent research by this agency did not identify a direct link between ESBL-positive strains of *E. coli* and chickens and humans (*18*), but other investigators found evidence for a link between drug resistance and specific genotypes of extratintestinal *E. coli* in animal food products and human infections in Minnesota and Washington, DC (*19–21*).

To further investigate the molecular epidemiology of disseminated *E. coli* clonal groups that cause UTIs, we conducted a cross-sectional study in a population of university women from Montréal, Québec, Canada, with UTI caused by *E. coli* and compared these organisms with those isolated from women with UTI in California. We sought to identify women in similar risk groups, but at different times and in different locations, to determine whether unrelated women with UTIs caused by indistinguishable strains of *E. coli* could be identified, and to determine whether the distribution was identical of clonal groups that were causing UTIs in these 2 communities.

Methods

Study Design

We conducted a cross-sectional study in collaboration with the Student Health Services at McGill University in Montréal in 2006. Eligible women 18–45 years of age who came to the health center with a suspected UTI were enrolled in the study. A UTI was clinically defined as $\geq$2 symptoms suggestive of this infection and included dysuria, increased urinary frequency or urgency, pyuria, hematuria, and $>10^2$ CFUs of *E. coli*/mL of clean-catch urine. If a woman had $\geq$1 UTIs during the study period, only data concerning the first UTI was eligible for inclusion in the analyses. Details of studies in California have been reported (*11*,*14*). The study protocol was reviewed and approved by the McGill University, Institutional Review Board (A01-M04–05A).

Isolation of *E. coli*

Urine samples were immediately cultured on Uricult (Orion Diagnostica, Espoo, Finland) MacConkey/cysteine lactose electrolyte–deficient agar dip slides. One arbitrarily selected colony (or multiple if morphologically different colonies were present) was selected from the MacConkey side. Lactose- and indole-positive colonies were presumptively identified as *E. coli* (*22*). Those isolates that were either lactose or indole negative were cultured on CHROMagar orientation plates (Becton Dickinson BBL Diagnostics, Sparks, MD, USA) and tested for lysine and ornithine decarboxylases (Moeller decarboxylase tests; PML Microbiologicals, Mississauga, Ontario, Canada). The reference strains used for carboxylase testing included *Klebsiella pneumoniae* (American Type Culture Collection [ATCC] no. 13883) and *Enterobacter cloacae* (ATCC no. 13047). Those isolates that were classified as *E. coli* on the CHROMagar plates and positive for lysine and ornithine decarboxylases were presumptively identified as *E. coli*. One *E. coli* isolate from each urine culture was arbitrarily selected for further analysis.

Antimicrobial Drug Susceptibility

Isolates were screened for susceptibility to TMP-SMZ, ciprofloxacin, cephalothin, nitrofurantoin, ampicillin, chloramphenicol, streptomycin, and tetracycline by the disk diffusion assay (Becton Dickinson BBL Diagnostics). *E. coli* strain ATCC 25922 was used as the reference strain. Isolates were defined as resistant, intermediate, or susceptible to each antimicrobial drug according to Clinical and Laboratory Standards Institute interpretive criteria (*23*). Isolates with intermediate resistance were defined as susceptible.

ERIC2 PCR Fingerprinting

All *E. coli* isolates were screened by using the ERIC2 PCR fingerprinting assay (*24*). Images of electrophoretic patterns were scanned into a software program (GelCompar II version 3.5; Applied Maths Inc., Austin, TX, USA) for analysis. Dendrograms based on ERIC2 PCR patterns were inferred from the Dice similarity coefficient matrix generated by GelCompar by the unweighted pair group method with arithmetic averages. Isolates with fingerprints that were indistinguishable on visual inspection or by GelCompar II version 3.5 (Applied Maths Inc.) analysis were grouped and selected for further typing.

Pulsed-Field Gel Electrophoresis

*Xba*I and *Not*I pulsed-field gel electrophoresis (PFGE) was conducted on all putative clonal isolates, as defined

by ERIC2 PCR (*25*). Isolates showing <6 band differences in their patterns were considered to be possibly related according to the criteria of Tenover et al. (*26*). Images of patterns were scanned into GelCompar II version 3.5 and analyzed as for ERIC2 PCR.

Serotypes

Serotyping was performed for Montréal *E. coli* isolates that were indistinguishable by ERIC2 PCR. O and H serotyping was performed by the Enteric Diseases Program at the National Microbiology Laboratory, Winnipeg, Manitoba, Canada, by using established protocols. Isolates from California were evaluated for serogroup only at the *E. coli* Reference Center (Pennsylvania State University, University Park, PA, USA). Isolates that were motile but non reactive with O or H antiserum were classified as nontypeable (OUNTYPE) and those that were nonmotile were denoted (HNM).

Multilocus Sequence Typing and Determination of Phylogenetic Group

Multilocus sequence typing (MLST) was performed as described (*27*). Gene amplification and sequencing were performed by using the primers specified at the *E. coli* MLST website (http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli). Allelic profile and sequence type (ST) determinations were assigned according to the *E. coli* MLST website scheme. The major *E. coli* phylogenetic group (A, B1, B2, and D) was determined by using a multiplex PCR (*28*).

Clonal Group

A clonal group was defined as ≥2 *E. coli* isolates showing indistinguishable patterns by ERIC2 PCR. These groups were given letter designations, such as CgA. Clonal group designations assigned for the California study isolates were retained (CgA to CgG), and clonal groups identified in Montréal were assigned new letter designation beginning with CgH. To support categorization of these clonal groups, isolates showing indistinguishable ERIC2 PCR patterns were also evaluated by PFGE, serotyping, drug susceptibility testing, MLST, and phylogenetic typing.

Statistical Analyses

All analyses were conducted by using Stata version 9.0 (Stata Corporation, College Station, TX, USA). Proportions and 95% confidence intervals (CIs) were estimated. Differences in proportions were assessed by χ^2 tests. Statistical significance was defined by $p<0.05$.

Results

Study Participants

From January 2006 to January 2007, 656 urine samples were submitted. *E. coli* was isolated from 300 urine samples obtained from 256 women in Montréal. Only samples from the first UTI were included in the analyses. A total of 311 (47%) samples yielded no bacteria, and 45 (7%) contained an organism other than *E. coli*. Results for the *E. coli* isolated from these 256 women with UTIs were compared with results for *E. coli* isolated from 434 women with UTIs in California (1999–2001).

Antimicrobial Drug Susceptibility

Antimicrobial drug resistance for the Montréal and California isolates is summarized in Table 1. For the drugs tested, isolates from Montréal showed comparable resistance levels to those from California, although resistance to TMP-SMZ was higher in isolates from California (20% in California vs. 14% in Montréal; p = 0.07) and ciprofloxacin resistance was slightly higher in isolates from Montréal (2% in California vs. 4% in Montréal; p = 0.06). Resistance to nitrofurantoin was not detected in isolates from either location.

ERIC2 PCR Fingerprinting

ERIC2 PCR fingerprinting identified 4 clonal groups (CgA, CgC, CgH, and CgI) among Montréal isolates (data not shown). The prevalence of these clonal groups in Montréal in 2006 was 13 CgA (5%, 95% CI 0.03–0.09), 10 CgC (4%, 95% CI 0.02–0.07), 7 CgI (3%, 95% CI 0.01–0.06), and 5 CgH (2%, 95% CI 0.01–0.04). CgA and CgC were identified from both study sites. In the California studies, 32 CgA isolates (7%, 95% CI 0.05–0.10) and 12 CgC isolates (3%, 95% CI 0.01–0.05) were identified. Clonal groupings were confirmed by PCR reamplification, and these groupings also included representatives of clonal groups identified in the California studies (*11*,*14*).

CgH was uniformly resistant to ampicillin and streptomycin and susceptible to all other drugs tested. CgC was susceptible to all drugs tested (except for 1 isolate that was resistant to ampicillin). CgA was primarily resistant to TMP-

Table 1. Antimicrobial drug resistance of *Escherichia coli**

Characteristic	Berkeley, California, USA†	Montréal, Québec, Canada‡	p value§
Total primary *E. coli*	434	256	
Drug	No. (%) resistant		
Trimethoprim-sulfamethoxazole	85 (20)	36 (14)	0.07
Cephalothin	11 (3)	7 (3)	0.90
Ciprofloxacin	8 (2)	11 (4)	0.06
Nitrofurantoin	0	0	
Ampicillin	ND	83 (32)	ND
Tetracycline	ND	40 (16)	ND
Chloramphenicol	ND	7 (3)	ND
Streptomycin	ND	48 (19)	ND

*ND, not done.
†October 1999–January 2000 and October 2000– January 2001.
‡January 2006–January 2007
§By χ^2 test.

SMZ and ampicillin; resistance to the other drugs varied. CgI showed the most extensive resistance. This group was resistant to ciprofloxacin and TMP-SMZ, and 2 members of CgI were resistant to 5 drugs. Drug-resistance profiles for each clonal group member from both study sites are shown in Table 2 and the online Appendix Table (available from www.cdc.gov/EID/content/14/10/1575-appT.htm).

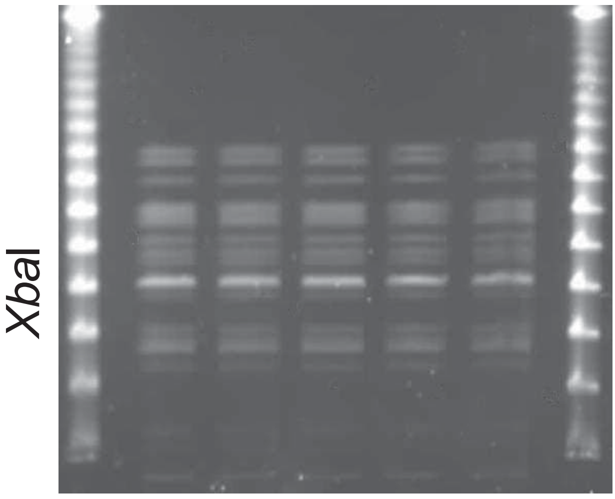
Pulsed-Field Gel Electrophoresis

PFGE confirmed the presence of 4 clonal groups among the Montréal isolates. CgH was found only in Montréal and showed indistinguishable *Xba*I and *Not*I PFGE patterns (Figure 1). CgI was also found only in Montréal and could be considered possibly related by the criteria of Tenover et al. (*26*) (Figure 2). Patterns of CgC isolates (Figure 3) identified in California and Montréal differed by <6 bands, regardless of restriction enzyme used. The PFGE results for CgA varied the most among all clonal groups from Montréal; in some cases, the PFGE patterns showed >6 band differences (Figure 4).

Table 2. Characteristics of clonal isolates of *Escherichia coli* from women with urinary tract infections, Montréal, Québec, Canada, 2006

Isolate no.	Genotype*	Serotype	MLST†	Phy‡	Date of infection	Antimicrobial drug resistance profile§							
						CIP	CEP	NIT	TMP-SMZ	AMP	CAM	STR	TET
362	C	O1:H7	ST95	B2	2006 Jan 23	0	0	0	0	0	0	0	0
363	C	O1:H7			2006 Jan 23	0	0	0	0	0	0	0	0
413	C	O18:H7	ST95	B2	2006 Feb 13	0	0	0	0	0	0	0	0
414	C	O1:H7			2006 Feb 13	0	0	0	0	0	0	0	0
439	C	O1:H7			2006 Feb 28	0	0	0	0	0	0	0	0
762	C	O1:K1:H7	ST95	B2	2006 Sep 28	0	0	0	0	0	0	0	0
767	C	O1:K1:H7			2006 Sep 29	0	0	0	0	1	0	0	0
782	C	O2:K1:H7	ST95	B2	2006 Oct 10	0	0	0	0	0	0	0	0
957	C	O1:H7			2007 Jan 1	0	0	0	0	0	0	0	0
958	C	O1:H7	ST95	B2	2007 Jan 5	0	0	0	0	0	0	0	0
412	H	O6:H1	ST73	B2	2006 Feb 13	0	0	0	0	1	0	1	0
415	H	O6:H1			2006 Feb 13	0	0	0	0	1	0	1	0
422	H	O6:H1			2006 Feb 16	0	0	0	0	1	0	1	0
459	H	O6:H1			2006 Mar 10	0	0	0	0	1	0	1	0
471	H	O6:H1			2006 Mar 16	0	0	0	0	1	0	1	0
385	A	OR:H18	ST69	D	2006 Jan 30	0	0	0	0	1	0	1	0
434	A	O73:H18	ST69	D	2006 Feb 27	0	0	0	0	0	0	0	0
498	A	O77/17:H18	ST69	D	2006 Mar 24	0	0	0	1	1	0	1	0
713	A	OUNTYPE: HNM	ST69	D	2006 Sep 11	0	0	0	1	1	0	1	0
724	A	O15:H18	ST69	D	2006 Sep 13	0	0	0	0	0	0	0	0
799	A	OUNTYPE: H18	ST69	D	2006 Oct 16	0	0	0	1	1	0	1	0
839	A	O17:H18	ST69	D	2006 Nov 2	0	0	0	1	0	0	0	0
860	A	O25:H18	ST69	D	2006 Nov 11	0	0	0	0	0	1	0	1
868	A	OUNTYPE: H18	ST69	D	2006 Nov 15	0	0	0	0	0	0	0	0
908	A	O17:H18	ST69	D	2006 Nov 30	0	0	0	1	1	0	0	1
912	A	O17:H18	ST69	D	2006 Nov 30	0	0	0	1	1	0	1	0
913	A	O17:HNM	ST69	D	2006 Dec 1	0	0	0	0	0	0	0	1
956	A	OUNTYPE: H18	ST69	D	2007 Jan 3	0	0	0	0	0	1	1	1
375	I	O25:H4	ST131	B2	2006 Jan 25	1	0	0	0	1	0	0	0
452	I	O25:H4	ST131	B2	2006 Mar 8	1	0	0	1	1	0	0	1
544	I	O25:H4			2006 Apr 19	1	0	0	0	0	0	0	0
550	I	O25:HNM	ST131	B2	2006 Apr 20	1	0	0	1	1	0	1	1
760	I	O25:H4			2006 Sep 28	1	0	0	1	1	0	0	1
783	I	O25:H4			2006 Oct 11	1	0	0	0	1	0	0	0
841	I	O25:H4	ST131	B2	2006 Nov 3	1	0	0	1	1	0	1	1

*Determined by ERIC2 PCR (*24*).
†MLST, multilocus sequence typing, according to Tartof et al. (*27*); ST, sequence type.
‡Phy, phylogenetic group, determined by multiplex PCR (*28*).
§0, sensitive; 1, resistant, according to Clinical and Laboratory Standards Institute interpretative criteria (*23*). CIP, ciprofloxacin; CEP, cephalothin; NIT, nitrofurantoin; TMP-SMZ, trimethoprim-sulfamethoxazole; AMP, ampicillin; CAM, chloramphenicol; STR, streptomycin; TET, tetracycline.



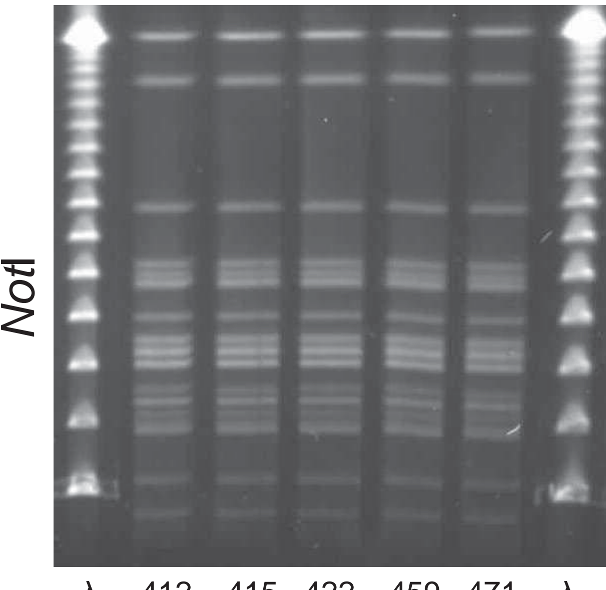


Figure 1. *Xba*I and *Not*I pulsed-field gel electrophoresis patterns for clonal group H *Escherichia coli* isolated from women with urinary tract infections in Montréal, Québec, Canada, 2006. The 5 isolates shown were serogroup O6:H1. First and last lanes, bacteriophage λ.

Serotypes

Serotype results for all clonal *E. coli* isolates identified in California and Montréal are shown in Table 2 and the Appendix Table. Serotyping was consistent within each clonal group, except for CgA, which showed 6 serogroups (O11, O77, O17, O73, O25, and O15) although O25 and O15 occurred only once. The complete serotype for CgA was O11/O17/O77/O73:K52:H18. CgC from both study locations showed the same serotype (O1/O18/O2:K1:H7).

MLST

Sequence types for selected members of each clonal group from the California and Montréal studies were determined (Table 2; Appendix Table). All sequence types were internally consistent within the clonal group. CgC and CgA isolates from both study sites showed the same sequence types (ST95 and ST69, respectively). CgH, CgB, and CgD showed the same sequence type (ST73). These 3 clonal groups also showed similar serogroups and phylogenetic groups but showed variable ERIC2 PCR and PFGE patterns; thus, they were not placed in the same clonal grouping.

Phylogenetic Group

Phylogenetic group was determined for selected members of each clonal group (Table 2 and Appendix Table). All phylogenetic group assignments were internally consistent within the clonal group and classified as either phylogenetic group B2 or D; both are typically associated with extraintestinal *E. coli*.

Time Cluster Analyses

In considering the hypothesis of endemic versus epidemic transmission of these clonal groups, temporal clus-

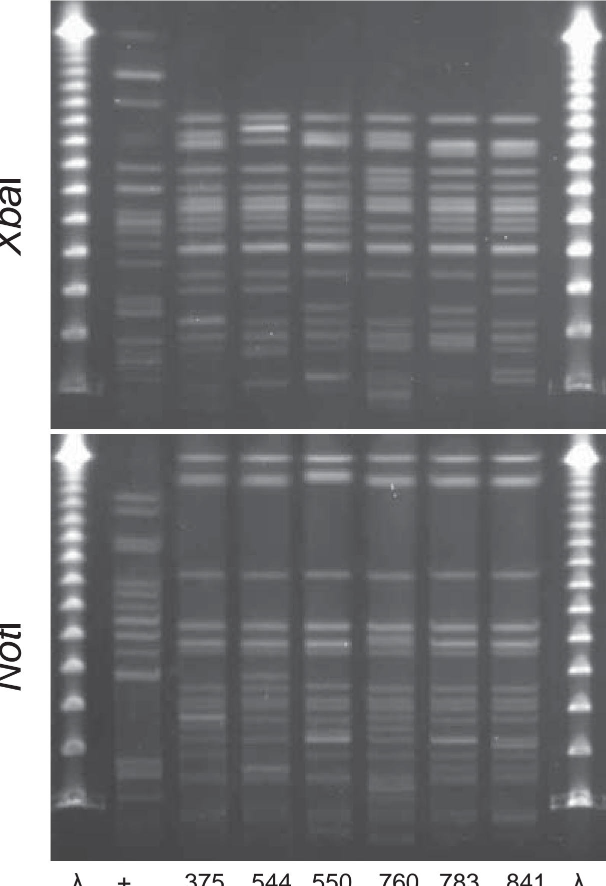


Figure 2. *Xba*I and *Not*I pulsed-field gel electrophoresis patterns for clonal group I *Escherichia coli* isolated from women with urinary tract infections in Montréal, Québec, Canada, 2006. The 6 isolates shown were resistant to ciprofloxacin and in serogroup O25:H4. First and last lanes, bacteriophage λ; lane +, positive control.

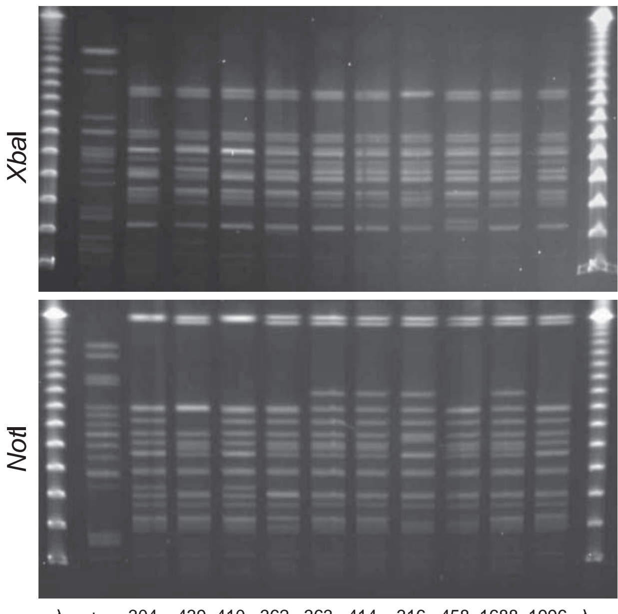


Figure 3. *Xba*I and *Not*I pulsed-field gel electrophoresis patterns for clonal group C *Escherichia coli* isolated from women with urinary tract infections in Montréal, Québec, Canada, 2006 (lanes 304, 439, 362, 363, and 414) and Berkeley, California, USA, 1999–2001 (lanes 410, 316, 458, 1688, and 1996). The 10 isolates shown were susceptible to all antimicrobial drugs tested and included serogroups O1, O2, or O18. First and last lanes, bacteriophage λ; lane +, positive control.

tering is a useful factor. Figure 5 shows the temporal pattern by week of UTI cases for all clonal groups in Montréal (Figure 5, panel A) and in California (Figure 5, panel B). Fluctuation in the number of *E. coli* UTIs over time corresponds closely to observation of clonal group–associated UTI cases. These results show clustering of some clonal groups, e.g., 3 of the 5 UTIs caused by CgH occurred in Montréal during week 7, and CgH did not appear again in Montréal after week 11. In California, CgA was present more frequently between October 1999 and February 2000 and dropped by 39% between the 2 sampling periods (*14*). CgB and CgD occurred exclusively in the second phase of the California study (Figure 5, panel B). Other clonal groups appeared throughout the year, although they often clustered by week. CgC was present during both data collection periods in California and caused UTIs throughout 2006 in Montréal. No clonal group members were identified during the summer in Montréal. However, this period corresponded to a decrease in the number of UTI cases at the student health services because of lower summer university enrollment (see total *E. coli* UTI by week, Figure 5).

Discussion

This study confirms the presence of drug-resistant, genetically related, and, in some cases, temporally clustered *E. coli* clonal groups (CgH, CgI, CgC, and CgA) that caused community-acquired UTIs in unrelated women in 2 locations and at different times. Drug resistance did not differ considerably between the 2 study sites, nor did the overall percentage of UTI caused by clonal groups: 4% (95% CI 0.10–0.18) in Montréal and 16% (95% CI 0.13–0.20) in California. Two clonal groups (CgA and CgC) were identified in both study locations, indicating widespread dissemination. These clonal groups shared common serogroups, PFGE patterns, drug-susceptibility profiles, MLST patterns, and phylogenetic groups. CgA isolates identified in Montréal did not show the same degree of genetic homogeneity as CgA isolates identified in the original California studies (*11,14*). CgA has also been

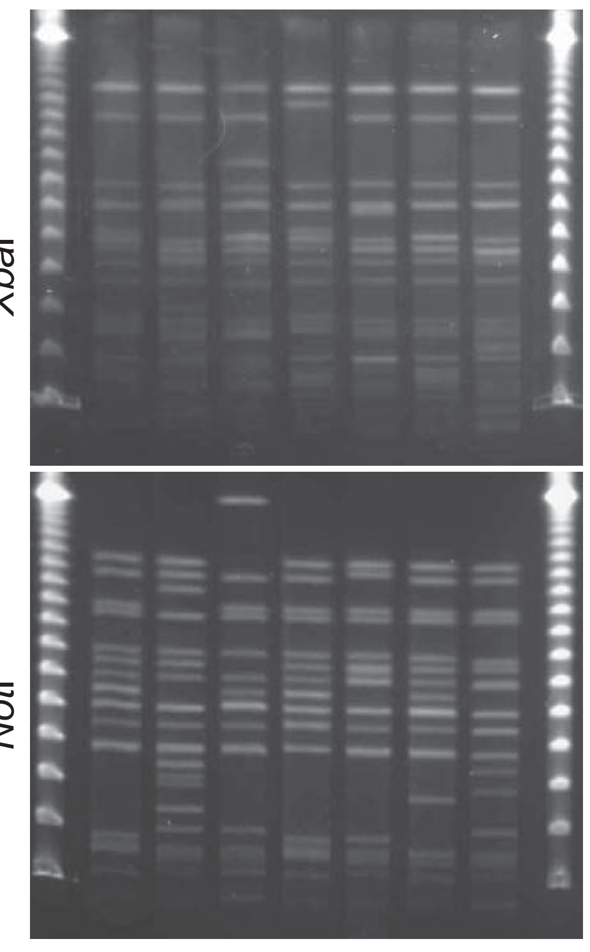


Figure 4. *Xba*I and *Not*I pulsed-field gel electrophoresis patterns for clonal group A *Escherichia coli* isolated from women with urinary tract infections in Montréal, Québec, Canada, 2006 (lanes 434 and 498) and Berkeley, California, USA, 1999–2001 (lanes 500, S203, W552, and S484). Antimicrobial drug resistance phenotypes and serogroups (O11, O17, O77, and O73) varied within and between the 2 study locations. First and last lanes, bacteriophage λ; lane +, positive control.

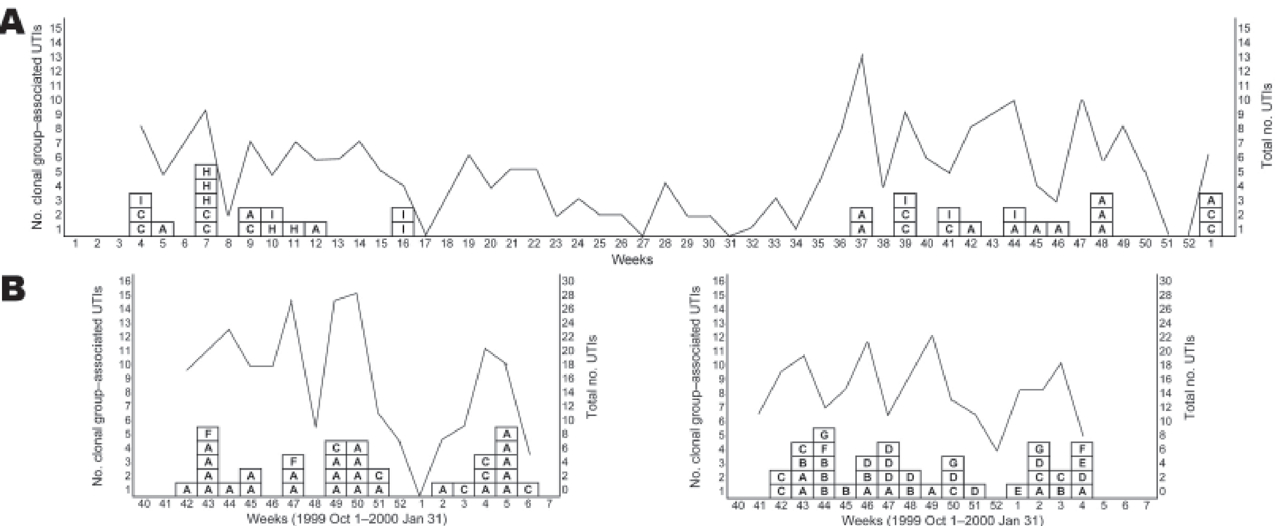


Figure 5. Temporal patterns of cases of urinary tract infections (UTIs) with *Escherichia coli* clonal groups by week in Montréal, Québec, Canada, 2006 (A), and Berkeley, California, USA, 1999–2001 (B). Clonal groups are identified by letters in boxes. Lines indicate the total number of UTIs with *E. coli* in each week for each study site. Samples were not analyzed during February–October 2000 in Berkeley.

recognized in many other locations and may represent a lineage that has been spreading over a longer period than other more genetically homogenous clonal groups identified (*6,11,14,29*). CgC members isolated from both study locations showed similar PFGE patterns, as well as common serotypes, MLST patterns, and phylogenetic groups, which suggest that these isolates are likely related. The fully susceptible nature of the CgC group and the similar drug resistance levels at the 2 study sites suggest that drug resistance or pressure may not have contributed to its selection and dissemination.

Of the 4 clonal groups, 3 showed resistance to ≥1 antimicrobial drugs. Most worrisome was CgI, which was resistant to ciprofloxacin and TMP-SMZ, drugs commonly used to treat patients with UTIs. Two members of CgI were resistant to 5 drugs. Identification of CgI serotype O25:H4 is also important because this serogroup and its drug resistance profile have been identified in a recent report on an emerging CTX-M type ESBL-producing *E. coli* (serotype O25:H4 and ST131) found worldwide (*30*). A possible link between the O25:H4 *E. coli* clonal group identified in Montréal and this emerging ESBL-producing *E. coli* clonal group should be investigated.

Temporal clustering (Figure 5) of these clonal groups from the 2 study sites was observed. Clonal groups tended to be identified in women on the same day and week or in adjacent weeks; CgH in Montréal and CgA in California followed this pattern. However, many of the clusters caused by these clonal groups did occur sporadically across the entire study period. The observed correlation between increased incidence of total *E. coli* UTIs and increased incidence of clonal group–associated UTIs may be a function of having sufficient numbers of UTIs to be able to detect these clonal groups of *E. coli*. However, underlying fluctuations in community-wide dynamics of these *E. coli* clonal groups (in a human or environmental reservoir) may influence the overall number of clinical infections.

One strength of our study is the ability to directly estimate the proportion of UTIs caused by each clonal group in the study communities. Because the study included all consecutive UTI specimens from a defined population and all *E. coli* were cultured and analyzed, it was possible to produce unbiased estimates of these proportions. Laboratory-based studies may overestimate prevalence of drug resistance, which in turn may bias the estimated proportion of clonal groups detected when specimens from recurrent, relapse, or complicated UTIs are disproportionately represented in the study samples.

One limitation of our study is the lack of epidemiologic data on possible *E. coli* transmission routes. Lack of epidemiologic information makes it impossible to determine what specific risk factors led groups of women to become infected with indistinguishable strains of *E. coli*. Therefore, detection of a specific transmission route (e.g., foodborne) could not be directly addressed in this study. However, an earlier study, on the basis of epidemiologic data, has implicated frequent consumption of chicken and pork in the development of drug-resistant UTIs (*31*). Also, limited reproducibility of the ERIC2 PCR may have contributed to an underestimation of the number of clonal groups, particularly those clonal groups with only a few members (*32*). However, additional genotypic and phenotypic analyses applied to these isolates contributed to the valid classification of these clonal groups.

Genetic homogeneity of the clonal groups identified in this study (CgH, CgC, and CgI), in addition to similar observations from other reports (*6,10,17*), suggests that these clonal groups are circulating in humans, most probably as part of the intestinal reservoir, and that they contribute to a sizable fraction of UTIs in the community. However, the degree of relatedness within each clonal group varied. For example, certain clonal groups (notably CgH) were highly clustered in time and showed indistinguishable genetic and other characteristics, which suggests local and recent transmission. Other clonal groups showed more diversity (e.g., CgA), possibly reflecting long-term, endemic transmission.

These results suggest 3 competing or coincident questions. First, do local, punctuated epidemics of specific strains or clonal groups occur as observed in this and earlier studies (*6,10,33*)? Second, do these clonal groups belong to a set of fairly conserved endemic clonal groups that are adapted for persistent and predominant colonization of the intestinal tract, and which have spread widely in human communities over varying periods of time (*6,10–14,29,33,34*)? Third, a combination of the first and second questions, are there periodic (epidemic) introductions of *E. coli* clonal groups in a community by an external source followed by endemic transmission? Already some evidence has indicated that animal-based foods or retail meats may contribute to the spread of these clonal groups (*19–21*). The number of infections, timing, and diverse locations in which these clonal groups are found argues against the possibility that person-to-person or household transmission contributes to our findings. However, limited local spread by these routes by certain clonal groups cannot be ruled out (*35–38*).

Positive and negative implications are associated with our results. One positive implication is that identification of lineages or clonal groups of *E. coli* that cause a sizeable fraction of community-acquired UTIs or extraintestinal infections may contribute to rational development of therapies and prevention strategies targeted toward these lineages. One negative implication is that tracing transmission routes and understanding the dynamics of these *E. coli* in external reservoirs and in human populations will be difficult and may impede possible control efforts, although ongoing attempts are under way to screen retail meats as a potential reservoir.

Annual incidence of UTIs and other community-acquired extraintestinal infections is high, in the millions, worldwide. Although each clonal group may account for a small fraction of all UTIs in a community, the high incidence of these infections implies that these clonal groups may contribute substantially to the overall extent of extraintestinal infections caused by *E. coli*. Furthermore, these clonal groups contribute, not only to uncomplicated infections such as cystitis, but also to severe infections such as pyelonephritis and septicemia (*13,39,40*). At a minimum, 10%–20% of these infections may be caused by 1 of a small set of extraintestinal pathogenic *E. coli* clonal groups, which are commonly resistant to $\geq$1 drugs. These facts point to the public health importance of understanding these *E. coli* lineages and their dynamics in the community and possible environmental reservoirs.

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Microbial Interactions during Upper Respiratory Tract Infections

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Learning Objectives

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

- Identify common bacterial isolates from children with upper respiratory infection
- Specify significant interactions between colonizing bacteria during upper respiratory infections
- Identify variables associated with higher rates of colonization with *Streptococcus pneumoniae*
- Specify which bacteria is more common in the nasopharynx of children who attend day care

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Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and *Staphylococcus aureus* often colonize the nasopharynx. Children are susceptible to bacterial infections during or soon after upper respiratory tract infection (URI). We describe colonization with these 4 bacteria species alone or in combination during URI. Data were from a prospective cohort of healthy children 6 to 36 months of age followed up for 1 year. Analyses of 968 swabs from 212 children indicated that *S. pneumoniae* colonization is negatively associated with colonization by *H. influenzae*. Competitive interactions shifted when *H. influenzae* and *M. catarrhalis* colonized together. In this situation, the likelihood of colonization with all 3 species is higher. Negative associations were identified between *S. pneumoniae* and *S. aureus* and between *H. influenzae* and *S. aureus*. Polymicrobial interactions differed by number and species of bacteria present. Antimicrobial therapy and vaccination strategies targeting specific bacterial species may alter the flora in unforeseen ways.

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Streptococcus pneumoniae, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* often asymptomatically colonize the nasopharynx of young children and are also associated with disease. *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* are the 3 most common otitis media pathogens (*1,2*). *S. pneumoniae* are also common causes of pneumonia, sepsis, and meningitis in young children (*3*). The proportion of young children colonized with any of these 3 bacteria species can be >50% in certain populations (*4–6*). *S. aureus* strains colonize up to 35% of young children and are associated with a wide range of diseases including soft tissue infections, sepsis, and pneumonia (*7,8*). Increases in the incidence of disease caused by community-acquired methicillin-resistant *S. aureus* are of great concern (*9*).

Host factors have been shown to influence colonization with *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. aureus*. These include host immunity, age, gender, race, out-of-home daycare, breastfeeding, and environmental exposure to tobacco smoke (*10*). The magnitude of host effects may differ by bacteria species.

Interactions between bacteria influence which species persist in the nasopharynx (*11–13*). Bacteria species may

be positively associated; this occurs when they are found together more often than would be expected by chance. A negative association could occur when bacteria compete within same environment. Several studies have described a negative association between *S. pneumoniae* and *S. aureus* (*12–16*). Understanding of interactions between *S. pneumoniae*, *H. influenzae*, *M. catarrhalis,* and *S. aureus* is limited.

The nasopharyngeal flora change over time; the level of bacteria colonization is higher during upper respiratory infection (URI) (*6,17*). Knowledge is lacking regarding *S. pneumoniae*, *H. influenzae*, *M. catarrhalis,* and *S. aureus* interactions during URI because colonization studies either do not examine competitive interactions among all 4 pathogens or focus on healthy children (*5,11,16,18*). Children are susceptible to secondary bacterial infections during and after URI (*19–21*).

A better understanding of polymicrobial interactions in the nasopharynx is important for several reasons. Colonization is the initial step in the disease process (*22, 23*). Colonized children serve as reservoirs for bacterial transmission to others in the community (*24*). Additionally, antibimicrobial drugs or vaccines, which target specific bacteria species, may alter polymicrobial interactions in the nasopharynx and have unanticipated consequences (*25,26*). The goals of our study were to 1) describe the prevalence of colonization with *S. pneumoniae, H. influenzae, M. catarrhalis*, and *S. aureus*; 2) evaluate interactions between *S. pneumoniae*, *H. influenzae, M. catarrhalis,* and *S. aureus*; and 3) estimate the effect of host factors on colonization with *S. pneumoniae*, *H. influenzae, M. catarrhalis*, and *S. aureus* after a URI in a prospective cohort of young children.

Methods

Study Design and Participants

We used data from a prospective study of otitis media complications of URI in children at the University of Texas Medical Branch (UTMB) at Galveston (*19,26*). The study was reviewed and approved by the UTMB Institutional Review Board. The parents of healthy children 6 months through 3 years of age, who were receiving medical care at UTMB from January 2003 through March 2007, were invited to enroll their children. Children with chronic medical problems and anatomic or physiologic defects of the ear or nasopharynx were excluded.

At enrollment, we collected information about demographic and URI risk factors. Parents were asked to describe their child's race and ethnicity. We also obtained information regarding the number of weeks the child had been breast-fed and the number of hours and days/week the child currently attended day care. We ascertained environmental exposure to tobacco smoke based on self-reports of whether any household members smoked cigarettes in the home.

The children in our study were followed up for 1 year. We requested that parents notify study staff when the child began to exhibit URI symptoms including nasal congestion, rhinorrhea, cough, sore throat, or fever. A study physician saw children as soon as possible after the onset of URI symptoms. At each study visit, the study physician obtained information regarding specific URI symptoms and examined the child's ears. The children were then monitored closely for 3 weeks for the development of otitis media. The study physician collected a nasopharyngeal swab during the visit for each URI episode and when acute otitis media or sinusitis was diagnosed. URI episodes were categorized as the same episode if symptoms persisted. An episode of URI was considered new when symptoms of the previous episode subsided and the parents noted new symptoms of URI as described above. Given our prospective study design, many children had >1 URI episode and some had >1 visit/URI episode. We collected 1 swab/physician visit. Data regarding antimicrobial drug therapy during the past 7 days were collected by medical record review. A description of the methods is provided elsewhere (*19,26*).

A total of 294 children were enrolled in the original study (*19,26*). Included in these analyses are data from 212 (72%) children who experienced at least 1 URI, were seen by a study physician, and had a nasopharyngeal swab collected for bacterial culture. Thus, we excluded 82 children who did not have a URI and a swab for bacterial culture. Of these 82 children without URI visits, 35 (59%) were lost to follow-up in the first 6 months, 13 (38%) were lost to follow-up in months 7–11, and 34 (17%) completed 1 year of follow up.

Mini-Tip Culturette kits (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) were used for sample collection. Each swab was streaked onto 1 blood and 1 chocolate agar plate. We subcultured and identified suspected isolates of each species as follows: *S. pneumoniae* isolates were identified by using the optochin disk susceptibility test (Taxo P, Becton Dickinson Microbiology Systems), *H. influenzae* isolates were identified by the *Haemophilus* ID Quad Plate with Growth Factors (Becton Dickinson Microbiology Systems), *M. catarrhalis* isolates were identified by the API QuadFerm assay (bioMérieux, Inc., Hazelwood, MO, USA), and *S. aureus* isolates were identified by coagulase, catalase, and latex agglutination test (Staphaurex Plus, Remel, Lenexa, KS, USA).

Statistical Methods

The main outcomes of interest were the relationships between bacteria during URI. All statistical analyses were

conducted by using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). We examined colonization by *S. pneumoniae*, *H. influenzae*, *M. catarrhalis,* and *S. aureus* by using repeated measures logistic regression with generalized estimating equations and an autoregressive correlation structure (AR1) using the procedure PROC GENMOD (SAS Institute, Inc.). Because each child could potentially have multiple URI episodes and contribute multiple bacterial swabs to the analysis, we used a repeated measures design to take into account variability of multiple samples from each child. To examine the effect of covariates on each bacteria species, we modeled colonization by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* separately. We did not separately model the outcome of colonization by *S. aureus* because of low numbers of isolates obtained. Each model included the presence or absence of other bacteria species, as well as potential sampling-time confounders comprising time of swab collection after URI onset, antimicrobial drug therapy within the past 7 days, and age of the child at the time of swab collection. Host factors included in the model were gender, race, day care, breast-fed for ≥4 months, and environmental exposure to tobacco smoke.

Results

Characteristics of the study participants are shown in Table 1. The median age of study participants was 12.0 months; mean age was 14.1 (SD 7.4) months. Most children were white, were cared for at home, and had not been breast-fed for ≥4 months. Children were followed up for a median of 12 months and a mean of 10.7 (SD 2.8) months.

Individual children contributed between 1 and 20 swab specimens each (mean [SD] and median of 4.6 [3.8] and 3.0 swabs, respectively) from 1 to 18 URI episodes each (mean [SD] and median of 4.0 [3.3] and 3.0 episodes, respectively). Overall, at least 1 of the 4 species was isolated from 841 of 968 swab samples (86.9%) from 212 children. Of the 968 swabs, *S. pneumoniae* was present in 441 (45.6%), *H. influenzae* was present in 314 (32.4%), and *M. catarrhalis* was the most common bacteria species identified in 611 (63.1%) swabs. *S. aureus* was relatively rare in comparison; 69 swabs (7.1%) were positive for this species. The distribution and colonization patterns of the 4 bacteria species by swab and number of URI visits are shown in Table 2.

Most swabs (849 [87.7%]) were collected within 7 days of URI onset; 119 (12.3%) were taken 8–30 days after URI onset. Of the 968 swab samples, only 54 (5.6%) were collected from children who had taken antimicrobial drugs within the past 7 days. Therefore, most swabs were collected from children who were not taking antimicrobial drugs at the time of swab collection (94.5%). Of the 212 children, 205 (>96%) had received at least 1 dose of the 7-valent pneumococcal conjugate vaccine (PCV7) at the time of enrollment. Most of the children had received all age-appropriate scheduled PCV7 vaccinations at their URI visit, 666 (69%) of samples were collected from children who had received the age-appropriate number of PCV7 doses at the time of swab collection. There was no association between being up to date with PCV7 vaccination and colonization with *S. pneumoniae* (p = 0.71). We did not further examine the effect of the pneumococcal vaccine further because of the high level of coverage in our study population.

Table 1. Characteristics of study participants enrolled through the University of Texas Medical Branch, Galveston, Texas, USA, 2003–2007*

Characteristic	No. (%)
Age at enrollment, mo	
6–<12	92 (43.4)
12–<18	62 (29.2)
18–<24	30 (14.2)
24–<36	28 (13.2)
Gender	
F	103 (48.6)
M	109 (51.4)
Race	
White	124 (58.5)
Black	62 (29.2)
Asian	6 (2.8)
Other	20 (9.4)
Ethnicity	
Hispanic or Latino	95 (44.8)
Not Hispanic	117 (55.2)
Day care†	
No	147 (69.7)
Yes	64 (30.3)
Breast-fed for ≥4 mo	
No	173 (82.0)
Yes	38 (18.0)
Environmental exposure to tobacco smoke‡	
No	145 (68.4)
Yes	67 (31.6)

*Data given for 212 participants who experienced at least 1 upper respiratory infection, were seen by a study physician, and had a nasopharyngeal swab collected for bacterial culture. An additional 82 enrollees were excluded from the study because they did not experience an upper respiratory infection and did not have a nasopharyngeal swab collected for bacterial culture. Some numbers do not add up to 212 because of missing data.
†No. hours and days/week in day care were grouped into any or none.
‡Environmental exposure to tobacco smoke was based on parental self-report.

Repeated measures logistic regression models predicting colonization by *S. pneumoniae, H. influenzae*, or *M. catarrhalis* are shown in Table 3. A positive association between bacteria is indicated by an odds ratio (OR) ≥1; a negative association is indicated by an OR <1. An OR of 1.0, or any 95% confidence interval that includes 1.0 indicates no significant association. The model predicting colonization by *S. pneumoniae* indicated that colonization by *H. influenzae* was negatively associated with *S. pneumoniae*. However, when *H. influenzae* and *M. catarrhalis* colonized together, they were positively associated with *S. pneumoniae* colonization. Colonization by *S. aureus* resulted in

Table 2. Distribution of bacteria on nasopharyngeal swabs collected from children with URI, University of Texas Medical Branch, Galveston, Texas, USA, 2003–2007*

Variable	Total no. (%) URI visits	No. (%) URI visits†				
		1	2	3–4	5–6	>6
Total no. patients	212	46 (21.7)	42 (19.8)	38 (17.9)	37 (17.4)	49 (23.1)
Total no. swabs	968	46 (4.8)	84 (8.7)	128 (13.2)	201 (20.8)	509 (52.6)
Bacteria present (% of no. of swabs in each visit category)						
0	127 (13.1)	9 (19.6)	9 (10.7)	13 (10.2)	19 (9.4)	77 (15.1)
1						
Streptococcus pneumoniae	79 (8.2)	1 (2.2)	9 (10.7)	15 (11.7)	20 (10.0)	34 (6.7)
Haemophilus influenzae	86 (8.9)	7 (15.2)	10 (11.9)	11 (8.6)	18 (9.0)	40 (7.9)
Moraxella catarrhalis	201 (20.8)	10 (21.7)	12 (14.3)	27 (21.1)	41 (20.4)	111 (21.8)
Staphylococcus aureus	24 (2.5)	1 (2.2)	2 (2.4)	2 (1.6)	3 (1.5)	16 (3.1)
2						
S. pneumoniae, *H. influenzae*	28 (2.9)	1 (2.2)	2 (2.4)	4 (3.1)	8 (4.0)	13 (2.6)
S. pneumoniae, *M. catarrhalis*	187 (19.3)	13 (28.3)	20 (23.8)	24 (18.8)	36 (17.9)	94 (18.5)
S. pneumoniae, *S. aureus*	8 (0.8)	0	1 (1.2)	1 (1.0)	4 (2.0)	2 (0.4)
H. influenzae, *M. catarrhalis*	67 (6.9)	2 (4.4)	5 (6.0)	7 (5.5)	13 (6.5)	40 (7.9)
H. influenzae, *S. aureus*	3 (0.3)	0	1 (1.2)	0	1 (0.5)	1 (0.2)
M. catarrhalis, *S. aureus*	17 (1.8)	0	2 (2.4)	3 (2.3)	2 (1.0)	10 (2.0)
3						
S. pneumoniae, H. influenzae, M. catarrhalis	124 (12.8)	2 (4.4)	8 (9.5)	19 (14.8)	31 (15.4)	64 (12.6)
S. pneumoniae, H. influenzae, S. aureus	2 (0.2)	0	1 (1.2)	0	0	1 (0.2)
S. pneumoniae, M. catarrhalis, S. aureus	11 (1.1)	0	1 (1.2)	2 (1.6)	4 (2.0)	4 (0.8)
H. influenzae, M. catarrhalis, S. aureus	2 (0.2)	0	0	0	1 (0.5)	1 (0.2)
4	2 (0.2)	0	1 (1.2)	0	0	1 (0.2)

*URI, upper respiratory tract infection.
†Data are presented as no. of physician visits/child. Because of our prospective study design, many children had >1 URI episode during the follow-up period, and some had >1 physician visit/URI episode. One nasopharyngeal swab sample was taken at each physician visit.

a 40% reduction in the odds of *S. pneumoniae* colonization. Older children were less likely to be colonized with *S. pneumoniae*; each 1-month increase in age was associated with a 2% decrease in the odds of *S. pneumoniae* colonization (Table 3). Antimicrobial drug therapy in the past 7 days was associated with decreased odds of *S. pneumoniae* colonization. The timing of swab collection after onset of URI symptoms and host characteristics such as gender, race, daycare, breastfeeding, and environmental exposure to tobacco smoke were not associated with colonization by *S. pneumoniae*.

In our model examining *H. influenzae* colonization as the outcome, *H. influenzae* was negatively associated with *S. pneumoniae, M. catarrhalis*, and *S. aureus* (Table 3). In contrast to their association with *S. pneumoniae* colonization, age and antimicrobial drug therapy during the past 7 days were not significantly associated with colonization by *H. influenzae*. Host characteristics were associated with colonization by *H. influenzae*. Male gender and out-of-home daycare were associated with increased odds of *H. influenzae* colonization. White race was associated with decreased odds of *H. influenzae* colonization.

Our third model examined factors associated with colonization by *M. catarrhalis* (Table 3). *H. influenzae* was negatively associated with colonization by *M. catarrhalis*, but when *H. influenzae* and *S. pneumoniae* colonized together, they were positively associated with colonization by *M. catarrhalis*. Older children were less likely to be colonized with *M. catarrhalis*; each 1-month increase in age was associated with a 2% decrease in the odds of *M. catarrhalis* colonization (Table 3). Antimicrobial drug therapy in the past 7 days was associated with decreased odds of *M. catarrhalis* colonization. The timing of swab collection after onset of URI symptoms and host characteristics such as gender, race, daycare, breastfeeding, and environmental exposure to tobacco smoke were not associated with colonization by *M. catarrhalis*.

Discussion

We describe nasopharyngeal colonization of children with *S. pneumoniae, H. influenzae, M. catarrhalis*, and *S. aureus* alone or in combination during URI. Our models predicting *S. pneumoniae* colonization indicated that *H. influenzae* is negatively associated with *S. pneumoniae*. However, when *H. influenzae* was present with *M. catarrhalis*, odds of *S. pneumoniae* colonization increased by >2-fold. Models predicting *H. influenzae* colonization indicated a negative association with *S. pneumoniae*, *M. catarrhalis*, and *S. aureus.* Competitive interactions between bacteria are complex after URI and may shift from negative to positive when additional bacteria species are present. Modeling *S. pneumoniae*, *H. influenzae,* and *M. catarrhalis* colonization separately showed that gender, race, and daycare were associated with colonization by *H.*

influenzae, but not with colonization by either *S. pneumoniae* or *M. catarrhalis*.

Jacoby et al. used a multivariate random effects model to examine *S. pneumoniae* colonization in Aboriginal and non-Aboriginal children in Australia (*11*). Their study differed from ours in that they examined the relationship between *S. pneumoniae, H. influenzae*, *M. catarrhalis,* and *S. aureus* in pairwise combinations. These researchers also examined healthy children; we examined children who had a URI. Jacoby et al. observed positive associations between pairwise combinations of *S. pneumoniae* and *H. influenzae* and between *S. pneumoniae* and *M. catarrhalis*. They did not identify an association between *S. pneumoniae* and *S. aureus* or between *H. influenzae* and *S. aureus* (*11*).

Our results confirm a recent report describing a negative association between *H. influenzae* and *S. aureus* in HIV-negative children (*12*). Our data also support a grow-

Table 3. Predicted outcome of colonization with *Stretococcus pneumoniae*, *Haemophilus influenzae*, or *Moraxella catarrhalis* in young children after upper respiratory tract infection (968 swabs from 212 children; see Table 2)*

Parameters	OR (95% CI)		
	S. pneumoniae	*H. influenzae*	*M. catarrhalis*
H. influenzae x *M. catarrhalis* (p = 0.0003)†			
Neither (reference)	1.0	–	–
H. influenzae only	**0.59 (0.40–0.88)**	–	–
M. catarrhalis only	1.31 (0.95–1.81)	–	–
Both	**2.13 (1.35–3.38)**	–	–
S. pneumoniae x *M. catarrhalis* (p = 0.08)†			
Neither (reference)	–	1.0	–
S. pneumoniae only	–	**0.52 (0.32–0.83)**	–
M. catarrhalis only	–	**0.45 (0.29–0.69)**	–
Both	–	0.82 (0.52–1.30)	–
H. influenzae x *S. pneumoniae* (p<0.0001)†			
Neither (reference)	–	–	1.0
H. influenzae only	–	–	**0.44 (0.30–0.63)**
S. pneumoniae only	–	–	1.22 (0.88–1.70)
Both	–	–	**2.09 (1.30–3.37)**
S. aureus			
Absent (reference)	1.0	1.0	1.0
Present	**0.60 (0.36–0.99)**	**0.36 (0.17–0.76)**	0.72 (0.42–1.25)
Age (1-mo increase)‡	**0.98 (0.96–1.00)**	1.01 (0.98–1.03)	**0.98 (0.97–1.00)**
Antimicrobial drug therapy in past 7 days			
No (reference)	1.0	1.0	1.0
Yes	**0.40 (0.22–0.72)**	1.21 (0.69–2.13)	**0.52 (0.28–0.96)**
Time after URI onset, d			
≤7 (reference)	1.0	1.0	1.0
>7	1.47 (0.96–2.27)	1.10 (0.70–1.73)	1.21 (0.81–1.80)
Gender			
F (reference)	1.0	1.0	1.0
M	1.05 (0.80–1.38)	**1.44 (1.08–.93)**	0.86 (0.65–1.14)
Race			
Not white (reference)	1.0	1.0	1.0
White	1.12 (0.84–1.48)	**0.42 (0.31–0.57)**	0.80 (0.60–1.07)
Day care			
No (reference)	1.0	1.0	1.0
Yes	1.32 (0.97–1.80)	**1.51 (1.09–2.09)**	1.09 (0.79–1.50)
Breast-fed ≥4 mo			
No (reference)	1.0	1.0	1.0
Yes	0.94 (0.69–1.29)	0.92 (0.65–1.29)	0.81 (0.59–1.12)
Environmental exposure to tobacco smoke			
No (reference)	1.0	1.0	1.0
Yes	1.13 (0.84–1.52)	0.93 (0.69–1.27)	0.91 (0.67–1.23)

*OR, odds ratio; CI, confidence interval. Significant ORs and 95% CIs are shown in **boldface**. Each model included variables representing presence or absence of other bacteria as well as all other variables listed. We did not model colonization of *S. aureus* because of low prevalence of this species (69/968 positive swabs).
†p value from logistic regression model for overall significance of bacterial interaction.
‡Age (mo) of the child at the time of swab collection.

ing body of literature describing negative associations between *S. pneumoniae* and *S. aureus* (*12–15*). For example, a cross-sectional study of 790 children younger than 40 months identified a negative association between *S. pneumoniae* colonization and *S. aureus* (OR 0.47; 95% confidence interval 0.28–0.78) (*13*).

An in vivo mouse model of competitive interactions between *S. pneumoniae* and *H. influenzae* has suggested mechanisms to explain our observations (*27*). Both *S. pneumoniae* and *H. influenzae* successfully colonized BALBc/SCID mice when each bacteria species was injected separately. However, *S. pneumoniae* was cleared rapidly when *H. influenzae* was present in a co-colonization model. The competitive interaction between *H. influenzae* and *S. pneumoniae* was dependent on complement and neutrophils (*27*). These researchers proposed that *H. influenzae* cellular components activate the host innate immune response, thus killing *S. pneumoniae* (*27*). *M. catarrhalis* was not examined in this model, but our data suggest that the additional presence of *M. catarrhalis* might alter the competitive balance between *S. pneumoniae* and *H. influenzae* and that all 3 bacteria species would successfully colonize.

In vitro studies have also demonstrated competition between *H. influenzae* and *S. pneumoniae* but predicted that *S. pneumoniae* should inhibit the growth of *H. influenzae*. Neuraminidase A is produced by *S. pneumoniae* and cleaves sialic acid. It has been shown to remove sialic acid from lipopolysaccharides of *H. influenzae* strains (*28*), potentially giving pneumococci a competitive advantage by making *H. influenzae* more susceptible to complement-mediated clearance. Furthermore, in vitro co-culture experiments indicate that *S. pneumoniae* can inhibit *H. influenzae* through the action of hydrogen peroxide (*29*). Interference between *S. pneumoniae* and *S. aureus* may also be caused by hydrogen peroxide production by *S. pneumoniae* (*30*).

Our results indicate that antimicrobial drug therapy in the past 7 days was associated with a lower prevalence of colonization with *S. pneumoniae* or *M. catarrhalis*. In contrast, antimicrobial drug therapy in the past 7 days was not associated with colonization by *H. influenzae*. Varon et al. studied the effect of antimicrobial drugs on colonization with *S. pneumoniae, H. influenzae*, and *M. catarrhalis* in a cohort of young children with URI (*31*). Children in this study received antimicrobial drugs for a mean treatment period of 8 days. Swab samples were taken before treatment and on days 2 through 6 after treatment. Results showed that colonization by *S. pneumoniae, H. influenzae*, and *M. catarrhalis* decreased after antimicrobial drug therapy (*31*). The magnitude of the effect differed by bacteria species and the specific antimicrobial drug prescribed. In general, antimicrobial drugs were less effective for reducing colonization with *H. influenzae* than with *S. pneumoniae* and *M. catarrhalis* (*31*).

The effect of age, gender, race, and breastfeeding on colonization differs by population studied (*10*). Daycare has consistently been associated with increased levels of colonization with *S. pneumoniae, H. influenzae*, and *M. catarrhalis* (*10*), as has exposure to other children in the household (*32,33*). Our study was limited by lack of data on age and number of siblings or other potential confounders such as household crowding and socioeconomic status.

Our study had additional limitations. A cross-sectional study of *S. aureus* and *S. pneumoniae* colonization indicated a negative association between PCV7 vaccine serotypes and *S. aureus* (*15*). No association was found between *S. pneumoniae* nonvaccine types and *S. aureus*. We were unable to examine the association between *S. pneumoniae* serotype and colonization. Along these lines, we did not have data regarding *H. influenzae* type B vaccination status and did not serotype our *H. influenzae* strains. Therefore, we were also unable to evaluate the effect of this vaccination on polymicrobial colonization.

Nasopharyngeal colonization likely involves a complex combination of factors including host characteristics that influence exposure to specific bacterial species, host immune responses that may result in killing the bacteria, and direct competitive interactions between bacteria species. In addition to the inhibiting effects of neuraminidase A and hydrogen peroxide already described, competitive interactions between bacteria may also include the secretion of small peptide inhibitors, competition for nutrients, and competition for receptor binding sites. It is also possible that the presence of 1 bacteria species could create a more hospitable niche for another bacteria species. We were unable to evaluate the precise molecular mechanisms that mediate these complex polymicrobial interactions, an important area for future research.

Our study had several strengths, including its longitudinal, prospective design. We examined nasopharyngeal carriage during URI, a time when children are at risk for secondary bacterial infections. In addition, we took advantage of repeated measures analytic techniques to examine microbe-level factors influencing bacterial colonization while controlling for host factors.

Results from our study have public health implications. Scientists have debated whether they should seek to eradicate disease by preventing nasopharyngeal colonization (*34*). Vaccines targeting nasopharyngeal carriage of *S. pneumoniae, H. influenzae*, and *M. catarrhalis* may be needed to prevent otitis media because simultaneous carriage of these 3 bacteria may increase risk for otitis media (*35*). Our data indicate that the elimination of nasopharyngeal colonization with bacteria such as *S. pneumoniae* and *H. influenzae* may increase risk for colonization with *S. aureus*. Scientists conducting a randomized trial of the effectiveness of pneumococcal vaccines noted an in-

crease in *S. aureus* when spontaneously draining infected middle ears of vaccinated children (*25*). Factors that may increase the risk of colonization with *S. aureus* are of special concern given the spread of methicillin-resistant *S. aureus* (*9*). Researchers are attempting to develop an *S. pneumoniae* vaccine containing pneumococcal choline binding protein A, which would protect against sepsis and pneumonia without interfering with pneumococcal colonization (*36*). Although this type of vaccination strategy may eventually decrease the incidence of potentially fatal invasive pneumococcal disease, it is unlikely to prevent otitis media. Thus, the public health impact of a given intervention strategy may be hard to predict, and caution should be used when designing control strategies that target nasopharyngeal colonization.

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Pyogenic Liver Abscess as Endemic Disease, Taiwan

Feng-Chiao Tsai, Yu-Tsung Huang, Luan-Yin Chang, and Jin-Town Wang

Pyogenic liver abscess has become a health problem in Taiwanese society. However, the extent of this problem has remained unclear because of the lack of a population-based study. We therefore performed a nationwide analysis of pyogenic liver abscess in Taiwan from 1996 through 2004. We analyzed 29,703 cases from the Taiwan National Health Insurance database and 506 cases from National Taiwan University Hospital. Our analysis showed that the annual incidence of pyogenic liver abscess increased steadily from 11.15/100,000 population in 1996 to 17.59/100,000 in 2004. Diabetes, malignancy, renal disease, and pneumonia were associated with a higher risk for the disease. By contrast, death due to pyogenic liver abscess decreased over time, although population-based abscess-related death increased slightly. Renal disease, malignancy, pneumonia, and heart disease correlated with higher death rates; *Klebsiella pneumoniae* infection and therapeutic procedures were related to lower death rates. Diabetes did not significantly change death rates for the 506 patients from the hospital.

The epidemiology of pyogenic liver abscess has changed dramatically in recent years (*1*). Previously, although incidence was considered rare, the condition was associated with high illness and death rates, usually due to underlying hepatobiliary diseases and polymicrobial infection (*2*), with *Escherichia coli* as the major pathogen (*3*). More recently, investigations in Taiwan suggest the role of cryptogenic or primary liver abscess, i.e., abscesses without underlying hepatobiliary diseases, in pyogenic liver abscess (*4,5*). These reports also indicate that diabetes is the major predisposing factor of liver abscess and that *Klebsiella pneumoniae* is the primary pathogen. However, these results were obtained from small-scale hospital-based surveys, which could not provide a panoramic view of the disease. To confirm these observation-based results, we conducted a large-scale, unbiased investigation.

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In addition to epidemiology, the pathogenesis of liver abscess caused by *Klebsiella* spp. has also been extensively studied, but the mechanism is still not clear. MagA, an outer-membrane protein contributing to capsular polysaccharide formation, coexists with serotype K1 and has been identified as the major virulence factor of *K. pneumoniae* (*6*). MagA-positive (or serotype K1) *K. pneumoniae* is accordingly recognized as the main pathogen of pyogenic liver abscess (*7,8*). Nevertheless, how diabetes increases the risk for *Klebsiella* spp. liver abscess is still not clear. Further research is needed on whether pyogenic liver abscess is affected by immunocompromised conditions, such as malignancy, renal failure, postorgan transplantation, or HIV infection.

To clarify the epidemiology and pathogenesis of pyogenic liver abscess, we used information gathered by the Taiwan National Health Insurance (NHI) program, which was initiated in 1995 by the government to cover most Taiwanese citizens. In 2005, 91.25% of healthcare providers were enrolled in the program and 99% of Taiwanese were insured (*9*). Consequently, since 1995, the program has obtained comprehensive health data on the population in Taiwan. In this study, we used NHI data to study the incidence and death rates caused by pyogenic liver abscess in Taiwan and to investigate factors modifying the manifestations of this disease.

Methods

Patients

We requested data on patients with pyogenic liver abscess from the Taiwan NHI program. Cases were selected by using the following criteria: patients were hospitalized and reported before 2004, and the discharge diagnoses included abscess of liver per the International Classification of Diseases, 9th revision, Clinical Modication (ICD-9-CM 572.0) but excluded amebic liver abscess (ICD-9-CM

006.3). Though we selected cases documented up to the end of 2004, the database could not provide information from patients who had not yet been discharged. Those admitted before December 31, 2004, but discharged during or after 2005 were therefore not included in our database. This exclusion results in the underestimation of case-patients admitted at the end of 2004.

Data on 29,965 case-patients were collected. After excluding patients discharged before 1996 and those without clear records regarding age or sex, we enrolled 29,703 case-patients in our study. Patient data were anonymous. Names of these patients were not included, and patient and health-care provider identification numbers were encrypted.

This primary set of data included the date of admission and discharge, age, sex, diagnoses (up to 5), procedures (up to 5), outcome at discharge (recovered or died), and the fees charged to patients. Laboratory data, including microbiologic data and medication, were not included. Any underlying diseases were determined by the diagnoses listed in the medical records, which were coded by ICD-9-CM.

Because *K. pneumoniae* is the major pathogen of primary pyogenic liver abscess in Taiwan, it is expected to play an important role in the pathogenesis and prognosis of this disease. Unfortunately, the NHI database does not include microbiologic data. To compensate for this, we reviewed the records of patients in National Taiwan University Hospital (NTUH). This hospital is a public medical center in Taipei, functioning both as a primary care hospital and as a tertiary referral center (*10*). As the leading hospital in Taiwan (*10*) with a 113-year history (*11*), NTUH serves patients and accepts referrals evenly distributed from every part of Taiwan. The hospital provides care for ≈2,000 inpatients and 7,000 outpatients a day (*11*), which are ≈3.5% and 2%, respectively, of persons included in the NHI database (*12*). Therefore, the patients of NTUH are representative of all of the patients in Taiwan, without substantial bias but may be skewed slightly to the severe side. We selected case-patients from this hospital using the same criteria mentioned above, except that the discharge year was between January 1, 2000, and December 31, 2004; complete microbiologic data was preserved in the NTUH laboratory only after 2000. These patients were included in the NHI database anonymously. For case-patients from NTUH, we reviewed actual medical records and obtained microbiologic data from the hospital's laboratory.

Statistical Analysis

Numerical data were compared by Student *t* test or paired *t* test. Categorical data were processed by χ^2 test. Pearson correlation coefficients and χ^2 goodness-of-fit test were used to estimate the trend of incidence and death over time. Unfortunately, incidence and death from different years could not be directly compared because the population structure changed slightly over the study period. To correct the bias, we calculated age-standardized incidence and death rates. The correction was based on age-specific population data in 1996. Finally, risk factor analysis was conducted by using the binary logistic regression and curve estimation methods by SPSS version 11.0 for Macintosh (SPSS, Inc. Chicago, IL, USA).

Results

Demographic Data

A total of 29,703 case-patients from the NHI database were enrolled in our analysis (Table 1). Ages of these patients ranged from <1 through 106 years of age, with a median age of 61 years; a total of 9,904 (33.3%) had diabetes mellitus, 3,079 (10.4%) had cirrhosis of the liver, 4,350 (14.6%) had cholelithiasis, and 4,115 (13.9%) had concomitant malignancy.

Average hospitalization was 17.33 days. The proportions of patients who received abscess drainage and biliary procedures (endoscopic or surgical biliary drainage) were 33.6% and 14.6%, respectively. The death rate was 10.9%.

Male patients dominated the sample population (18,326/29,703, 61.7%) and, on average, were 5 years younger than their female counterparts (57.58 ± 16.03 vs. 62.13 ± 14.87 years, $p<0.001$). Besides the difference in age, more female patients had concomitant cholelithiasis (18.6% vs. 12.2%, $p<0.001$). Female patients also received biliary procedures more often (17.3% vs. 12.9%, $p<0.001$) than did male patients.

We then investigated the data over time. An average of 3,300 cases/year or 275 cases/month were reported. The number of reported cases increased from 2,400 in 1996 to 3,991 in 2004 (Table 2), with a stable increase rate of 1.44 more new cases per month (Figure 1); the decline in cases at the end of 2004 is possibly due to the incomplete recruitment of cases as described in the Methods section.

The annual number of case-patients increased with age; peak incidence for women was seen in those 60–64 years of age and in men 65–69 years of age. A slight decline was noted in case-patients 55–59 years of age, a reflection of decreased birth rates during World War II. Men <85 years of age had more liver abscess than women but the opposite was seen in women >85 years of age.

Incidence and Risk Factors

The gross incidence of pyogenic liver abscess from 1996 through 2004 was 14.87 cases/100,000 population/year (17.94 male cases/100,000 population and 11.65 female cases/100,000 population). The annual increase of incidence was 0.86 cases/100,000 population (r = 0.98, $p<0.001$) (Figure 2, panel A). When we calculated age-standardized incidences, the increase rate was 0.51

Table 1. Demographic data from National Health Insurance database, Taiwan, 1996–2004

Item	Female	Male	Total	p value*
Sex, no. (%)	11,377 (38.3)	18,326 (61.7)	29,703 (100)	
Age: range (median), y	0–106 (63.90)	0–101 (58.98)	0–106 (61.00)	<0.001†
Age: mean (SD), y	62.13 (14.87)	57.58 (16.03)	59.32 (15.75)	<0.001‡
Hospitalization: mean (SD), d	18.00 (13.80)	16.91 (12.74)	17.33 (13.17)	<0.001‡
Diabetes mellitus, no. (%)	3,998 (35.1)	5,906 (32.2)	9,904 (33.3)	<0.001§
Cirrhosis, no. (%)	854 (7.5)	2,225 (12.1)	3,079 (10.4)	<0.001§
Renal disease, no. (%)	772 (6.8)	1,191 (6.5)	1,963 (6.6)	0.334§
Hypertension, no. (%)	1,491 (13.1)	1,866 (10.2)	3,357 (11.3)	<0.001§
Heart disease, no. (%)	826 (7.3)	1,118 (6.1)	1,944 (6.5)	<0.001§
Cerebrovascular accident, no. (%)	364 (3.2)	454 (2.5)	818 (2.8)	<0.001§
Cholelithiasis, no. (%)	2,121 (18.6)	2,229 (12.2)	4,350 (14.6)	<0.001§
Hepatobiliary malignancy, no. (%)	863 (7.6)	1,769 (9.7)	2,632 (8.9)	<0.001§
Other malignancy, no. (%)	546 (4.8)	937 (5.1)	1,483 (5.0)	0.227§
Pneumonia, no. (%)	675 (5.9)	1,273 (6.9)	1,948 (6.6)	0.001§
Urinary tract infection, no. (%)	923 (8.1)	697 (3.8)	1,620 (5.5)	<0.001§
Acute viral hepatitis, no. (%)	228 (2.0)	657 (3.6)	885 (3.0)	<0.001§
Peptic ulcer, no. (%)	985 (8.7)	1,623 (8.9)	2,608 (8.8)	0.557§
Abscess drainage no. (%)	3,886 (34.2)	6,082 (33.2)	9,968 (33.6)	0.082§
Biliary procedure no. (%)	1,967 (17.3)	2,361 (12.9)	4,328 (14.6)	<0.001§
Deaths, no. (%)	1,286 (11.3)	1,954 (10.7)	3,240 (10.9)	0.085§

*p<0.05 is considered statistically significant.
†Mann-Whitney U test.
‡Student *t* test.
§χ^2 test.

cases/100,000 population/year (r = 0.92, p<0.001) (Figure 2, panel B). The age of highest incidence in men was in those 80–84 years of age (86.71 cases/100,000 population); for women, the highest incidence was in those 85–89 years of age (79.80 cases/100,000 population).

Several factors were associated with the increased incidence of liver abscess. Because incidence data from each year could not be merged directly, we chose the cases reported in 2004 to evaluate the relative risk for each factor (Table 3). Diabetes mellitus and malignancy were associated with a ≈10-fold increased risk, while renal disease and pneumonia tripled and quadrupled the incidence of liver abscess, respectively. We did not investigate the interactions among these factors because it required detailed health records of each person in the Taiwan population, which would violate patient confidentiality regulations.

Change in Death Rates

In contrast to increased incidence rates from 1996 through 2004, the disease-specific death rate declined steadily from 12.33% in 1996 to 9.72% in 2004 while the number of deaths caused by pyogenic liver abscess increased slightly over this same timeframe (Figure 2, panel B). The yearly change in the death rate was –0.31% (r = 0.91, p<0.001) (Figure 3, panel A). When we calculated age-standardized death rates, the decrease was 0.38%/year (r = 0.94, p<0.001) (Figure 3, panel B). Death rates did not differ much between males and females (10.7% vs. 11.3%) (Table 1). Death rates increased slowly for men and women 35–85 years of age and peaked for those 90–94 years of age (33.81%). Minor peaks were noted for adolescents 10–14 years of age (19.35%) and for young men 20–24 years of age (12.36%), respectively.

Table 2. Incidence rates and deaths from pyogenic liver abscess, National Health Insurance database, Taiwan, 1996–2004*

	Case-patients with pyogenic liver abscess			Deaths from pyogenic liver abscess		
Year	Female	Male	Total	Female	Male	Total
1996	945 (9.03)	1455 (13.15)	2400 (11.15)	112 (1.07)	184 (1.66)	296 (1.38)
1997	1045 (9.88)	1562 (13.99)	2607 (11.99)	135 (1.28)	173 (1.55)	308 (1.42)
1998	1130 (10.58)	1701 (15.13)	2831 (12.91)	151 (1.41)	189 (1.68)	340 (1.55)
1999	1162 (10.78)	1936 (17.11)	3098 (14.02)	138 (1.28)	213 (1.88)	351 (1.59)
2000	1300 (11.94)	2094 (18.38)	3394 (15.24)	147 (1.35)	201 (1.76)	348 (1.56)
2001	1453 (13.25)	2218 (19.39)	3671 (16.38)	160 (1.46)	258 (2.25)	418 (1.87)
2002	1406 (12.74)	2485 (21.64)	3891 (17.28)	151 (1.37)	259 (2.26)	410 (1.82)
2003	1434 (12.93)	2386 (20.72)	3820 (16.90)	143 (1.29)	238 (2.07)	381 (1.69)
2004	1502 (13.47)	2489 (21.57)	3991 (17.59)	149 (1.34)	239 (2.07)	388 (1.71)
p value†	<0.001	<0.001	<0.001	0.224	<0.001	<0.001

*Data are presented as number (rate per 100,000 population).
†χ^2 goodness-of-fit test. p<0.05 is considered statistically significant.

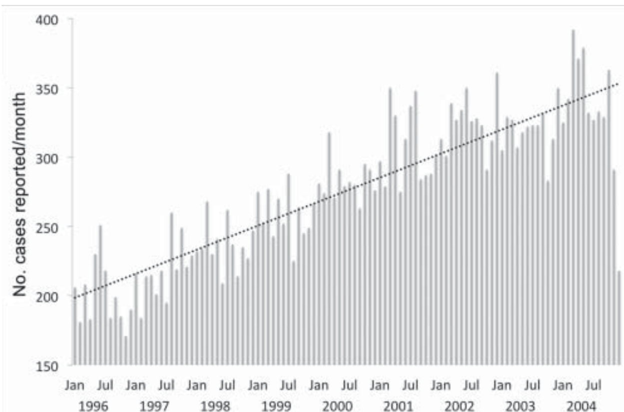

Figure 1. Number of new reported cases of pyogenic liver abscess recorded monthly in the National Health Insurance database, Taiwan, January 1996 through December 2004.

We tested case-patients with binary logistic regression to identify the factors contributing to increased death rates (Table 4). Multivariate analysis showed an increased risk of 1.7%/year of age. Renal disease and malignancy were respectively associated with a 2.5- and a 2-fold increased risk, followed by pneumonia (1.5-fold) and heart disease (1.3-fold). Some underlying diseases were associated with a lower risk for death, including diabetes (0.85-fold), peptic ulcer (0.75- fold), urinary tract infection (0.74-fold), hypertension (0.44-fold), and cholelithiasis (0.69-fold). Therapeutic procedures were also related to the lower risk of death (abscess drainage 0.57-fold, biliary drainage 0.73-fold), compatible with our expectations.

K. pneumoniae and Pyogenic Liver Abscess at NTUH

To compensate for the deficiency of microbiologic data in the NHI database, we reviewed the medical records of case-patients with primary pyogenic liver abscess admitted to NTUH from 2000 through 2004. In total, 506 case-patients were enrolled, ≈3.70% of all case-patients in Taiwan (13,672) during the same period. This ratio is similar to the general NTUH: Taiwan inpatient ratio (3.50%) (*12*), suggesting that liver-abscess cases in NTUH are probably a microcosm for the liver-abscess patients in all of Taiwan. Demographic data are shown in Table 5. Compared with the NHI database, case-patients in NTUH had longer hospitalization periods and higher frequencies of heart disease and malignancy, implying severe underlying conditions. However, the ratios of sepsis, meningitis, endophthalmitis, and pneumonia were similar between NTUH and Taiwan data, so the severity of primary liver abscess in NTUH patients and Taiwan as a whole were equal. Besides, NTUH case-patients were associated with higher rates of abscess drainage and lower death rates, indicating that the quality of medical care for those patients is probably better than the average level of medical care provided in Taiwan.

Among the 506 cases, 358 had positive culture results (Table 6), and 286 (79.9%) of 358 case-patients showed *K. pneumoniae* infection. Patients with *Klebsiella* spp. infection had a lower death rate (2.4% vs. 11.1%; $p = 0.004$), less mixed bacterial infection (4.5% vs. 26.4%; $p<0.001$), and less underlying malignancy (5.2% vs. 20.8%; $p<0.001$). Of case-patients with *Klebsiella* spp. liver abscess, 35% were associated with diabetes mellitus. The prevalence of diabetes in case-patients with other micromicrobial infections was 18.1% ($p = 0.007$). Binary logistic regression analysis showed that *Klebsiella* spp. infection was associated with decreased death rates (relative risk 0.20, $p = 0.003$); the role of diabetes was neutral (relative risk 1.09, $p = 0.88$) (Table 7). Therefore, the low death rates in case-patients with diabetes who also had liver abscess were probably attributed to *Klebsiella* spp. infection.

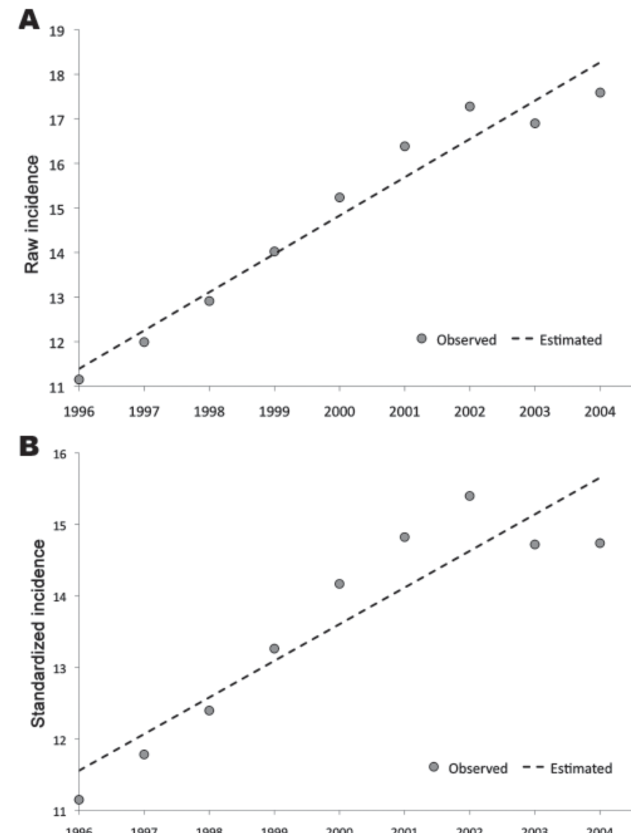

Figure 2. Incidence of pyogenic liver abscess in Taiwan, showing a steady increase from 1996 to 2004. The incidence is expressed as the number of new cases reported from the population (the National Health Insurance database) per year. A) The linear increase of the primary incidence data (raw incidence: cases/year × 1/100,000) with the year can be described by the formula Incidence × 1/100,000 = 0.860 × year – 1704.66 ($r = 0.978$, $p<0.001$). B) The linear increase of the standardized incidence data (raw incidence normalized according to the age distribution of the population) with the year can be described by the formula Incidence × 1/100,000 = 0.512 × year – 1010.68 ($r = 0.923$, $p<0.001$). *r*, Pearson correlation coefficient. $p<0.05$ is considered statistically significant.

Table 3. Factors associated with increased incidence in pyogenic liver abscess based on cases reported in the National Health Insurance database, Taiwan, 2004*

Factor	Incidence with factor	Incidence without factor	Relative risk (95% CI)
Sex (M)	21,565	13,474	1.601 (1.501–1.707)
Acute viral hepatitis	50,798	17,090	2.973 (2.511–3.466)
Diabetes mellitus	111,998	12,322	9.098 (8.520–9.716)
Malignancy	162,610	15,176	10.731 (9.840–11.702)
Hypertension	23,989	16,772	1.430 (1.313–1.559)
CVA	22,756	17,471	1.303 (1.083–1.567)
Pneumonia	77,048	16,541	4.661 (4.145–5.240)
Renal disease	62,795	16,878	3.722 (3.250–4.263)
Heart disease	19,494	17,468	1.116 (0.986–1.264)

*Incidence is expressed as cases/100,000 population. CI, confidence interval; CVA, cerebrovascular accident.

Discussion

We present a nationwide population-based report of pyogenic liver abscess in Taiwan. Taiwan started the NHI program in 1995, with a coverage rate of ≈99% of its population. By providing national healthcare, the system collected medical records from virtually every person seeking medical help in Taiwan so many nationwide surveys were automatically completed. The database, therefore, reflects a complete and unbiased picture of general health conditions in Taiwan.

Our data suggests Taiwan is endemic for pyogenic liver abscess. This disease had been considered rare in the past; previous reports showed an annual incidence rate of 2.3/100,000 (*13*) and 1.0/100,000 (*14*) in Canada and Denmark, respectively. In our report, however, the incidence rate was 10× higher (11.99/100,000 in 1996 and up to 17.59/100,000 in 2004). Such high incidence rates and rapid increase indicate the true growth of this disease because the data could not be explained solely by the change in population structure or by improved detection capacity of medical professionals. First, the diagnostic tools and methods for pyogenic liver abscess did not remarkably change during our study. Furthermore, the incidence did not explode but increased steadily over time. Thus, the increase is unlikely to have been caused by improved diagnostic sensitivity. Second, to eliminate bias induced by the change in population structure over time, we calculated age-standardized incidence. The increased incidence remains significant after correction (Figure 2, panels A, B). Hence, endemic pyogenic liver abscess possibly existed long before these data were first collected in Taiwan, even though the situation has recently become much worse. During the study period, magA-positive *K. pneumoniae* was identified as the major cause of pyogenic liver abscess in Taiwan (*6,15*). Although culture data were lacking in the NHI database, 80% of NTUH case-patients had positive culture data that showed *Klebsiella* spp. A recent nationwide report from South Korea also showed that the proportion of *K. pneumoniae* infection increased dramatically in that country over time from ≈0% in 1955–1969 up to 78.2% during 2004–2005 (*8*). We can therefore infer that the high incidence of pyogenic liver abscess in Taiwan is related to *Klebsiella* spp. infection.

In contrast to the rise of incidence over time, death rates from pyogenic liver abscess decreased. In our analysis, the death rate from pyogenic liver abscess in Taiwan was 10.9% during 1996–2004 (Table 1) while the inhospital death rate was 6.1% (Table 5). These rates are much lower than those in earlier reports, 25% (*16*) to 50% (*14*), but

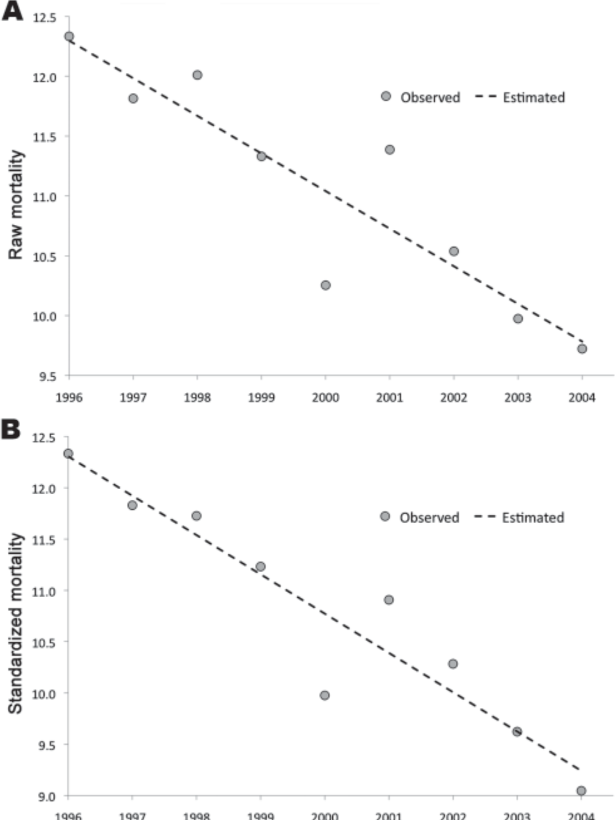

Figure 3. Deaths from pyogenic liver abscess in Taiwan, showing a steady decrease from 1996 to 2004. Mortality rate is expressed as the number of deaths reported from pyogenic liver abscess cases per year. A) The linear decrease of the primary mortality data (raw mortality: deaths/case/year × 1/100) with the year can be described with this formula: Mortality × 1/100 = 0.314 × year + 639.58 (r = 0.910, p<0.001). B) The linear decrease of the standardized mortality data (raw mortality normalized according to the age distribution of the population) with the year can be described with this formula: Mortality × 1/100 = 0.383 × year + 776.59 (r = 0.944, p<0.001). r, Pearson correlation coefficient. p<0.05 is considered statistically significant.

Table 4. Binary logistic regression analysis showing factors associated with death from pyogenic liver abscess, Taiwan, 1996–2004*

	Univariate analysis			Multivariate analysis		
Factor	Odds ratio	p value†	95% CI	Odds ratio	p value†	95% CI
Age, y	1.018	<0.001	1.015–1.020	1.017	<0.001	1.015–1.020
Sex (M)	0.937	0.085	0.869–1.009	0.930	0.067	0.861–1.005
Diabetes mellitus	0.813	<0.001	0.750–0.880	0.850	<0.001	0.782–0.925
Abscess drainage	0.541	<0.001	0.497–0.590	0.571	<0.001	0.523–0.624
Biliary procedure	0.635	<0.001	0.564–0.715	0.728	<0.001	0.640–0.828
Peptic ulcer	0.778	<0.001	0.676–0.895	0.751	<0.001	0.651–0.867
Urinary tract infection	0.851	0.063	0.718–1.009	0.735	0.001	0.617–0.875
Cirrhosis	1.027	0.663	0.912–1.156	–	–	–
Renal disease	2.599	<0.001	2.322–2.909	2.452	<0.001	2.181–2.756
Hypertension	0.480	<0.001	0.414–0.557	0.435	<0.001	0.374–0.506
Cerebrovascular accident	1.405	0.001	1.153–1.713	1.219	0.058	0.993–1.497
Cholelithiasis	0.599	<0.001	0.530–0.676	0.693	<0.001	0.608–0.790
Hepatobiliary malignancy	1.745	<0.001	1.564–1.947	1.850	<0.001	1.646–2.080
Other malignancy	2.151	<0.001	1.883–2.456	2.136	<0.001	1.859–2.454
Pneumonia	1.770	<0.001	1.563–2.003	1.516	<0.001	1.333–1.723
Acute viral hepatitis	0.481	<0.001	0.361–0.642	0.431	<0.001	0.321–0.578
Heart disease	1.384	<0.001	1.212–1.581	1.254	0.001	1.092–1.440

*CI, confidence interval.
†p<0.05 is considered statistically significant.

are consistent with those in more recent reports, in which population-based death rates were ≈10% (*13,14*) while in-hospital death rates ranged from 6%–8% (*15,17,18*). This decrease might be multifactorial.

First, the decrease in death rates might be the result of the dramatic increase of case-patients with pyogenic liver abscess. In a previous report, Jepsen et al. suggested that the dramatic decrease in death rates in Denmark between 1977 and 2002 resulted from improved diagnostic tools (*14*). This reason might also apply in Taiwan because the liver abscess–related death rate in the general population actually increased from 1.38/100,000 in 1996 to 1.80/100,000 in 2004. However, the increase was not steady, with a peak of 1.94/100,000 in 2001 followed by a decrease, indicating a true decrease of disease-specific deaths. Thus, diagnostic sensitivity could not explain the whole condition.

Second, the advance of medical care might have contributed to the decrease in mortality rates. Because abscess

Table 5. Demographic data on patients with primary pyogenic liver abscess from NTUH and NHI databases, Taiwan, 2000–2004*

Item	NHI database	NTUH	p value†
Total no. case-patients	13,672	506 (3.70)	
Age, y, range (median)	0–100 (59.95)	0–90 (57.50)	
Age, y, mean (SD)	58.31 (16.37)	55.92 (16.93)	0.001
Hospitalization, mean (SD)	17.32 (12.80)	30.13 (34.08)	<0.001
M/F, no. (%)	8,652/5,020 (63.3/36.7)	329/177 (65.0/35.0)	0.426
Diabetes mellitus, no. (%)	5,428 (39.7)	140 (27.7)	<0.001
Cirrhosis, no. (%)	1,153 (8.4)	19 (3.8)	<0.001
Renal disease, no. (%)	1,113 (8.1)	47 (9.3)	0.355
Hypertension, no. (%)	1,947 (14.2)	88 (17.4)	0.047
Heart disease, no. (%)	1,038 (7.6)	53 (10.5)	0.017
Cerebrovascular accident, no. (%)	461 (3.4)	17 (3.4)	0.988
Other malignancy, no. (%)	930 (6.8)	77 (15.2)	<0.001
Sepsis, no. (%)	3,252 (23.8)	137 (27.1)	0.089
Meningitis, no. (%)	155 (1.1)	6 (1.2)	1.000
Endophthalmitis, no. (%)	226 (1.7)	10 (2.0)	0.593
Pneumonia, no. (%)	1,141 (8.3)	54 (10.7)	0.064
Urinary tract infection, no. (%)	916 (6.7)	20 (4.0)	0.015
Acute viral hepatitis, no. (%)	400 (2.9)	10 (2.0)	0.211
Peptic ulcer, no. (%)	1,161 (8.5)	20 (4.0)	<0.001
Abscess drainage, no. (%)	4,795 (35.1)	292 (57.7)	<0.001
Biliary procedure, no. (%)	790 (5.8)	15 (3.0)	0.007
Deaths, no. (%)	1,410 (10.3)	31 (6.1)	0.002

*Data expressions and statistical methods in this table are the same as in Table 1. NTUH, National Taiwan University Hospital; NHI, National Health Insurance.
†p<0.05 is considered statistically significant.

Table 6. *Klebsiella* spp. liver abscess compared with other primary pyogenic liver abscess, NTUH, Taiwan, 1996–2004*

Item	*Klebsiella* spp.	Non-*Klebsiella* spp.	p value†
Total no.	286	72	
Male gender, no. (%)	191 (66.8)	45 (62.5)	0.490
Deaths, no. (%)	7 (2.4)	8 (11.1)	0.004
Abscess drainage, no. (%)	204 (71.3)	49 (68.1)	0.664
Biliary procedure, no. (%)	2 (0.7)	9 (12.5)	<0.001
Mixed infection, no. (%)	13 (4.5)	19 (26.4)	<0.001
Diabetes mellitus, no. (%)	100 (35.0)	13 (18.1)	0.007
Peptic ulcer, no. (%)	10 (3.5)	7 (9.7)	0.055
Urinary tract infectio, no. (%)	14 (4.9)	2 (2.8)	0.749
Renal disease, no. (%)	25 (8.7)	6 (8.3)	1.000
Hypertension, no. (%)	55 (19.2)	14 (19.4)	1.000
Heart disease, no. (%)	28 (9.8)	9 (12.5)	0.517
Cerebrovascular accident, no. (%)	7 (2.4)	3 (4.2)	0.427
Malignancy, no. (%)	15 (5.2)	15 (20.8)	<0.001
Cirrhosis, no. (%)	5 (1.7)	2 (2.8)	0.632
Pneumonia, no. (%)	35 (12.2)	6 (8.3)	0.414
Viral hepatitis, no. (%)	4 (1.4)	1 (1.4)	1.000
Meningitis, no. (%)	3 (1.0)	0 (0.0)	0.613
Endophthalmitis	9 (3.1)	0 (0.0)	0.214
Age, y, mean (SD)	57.39 (15.52)	55.25 (19.26)	0.384
No. hospitalizations, mean (SD)	26.12 (16.36)	43.06 (69.64)	0.044

*NTUH, National Taiwan University Hospital. The χ^2 test and Student *t* test were used for statistical analyses.
†p<0.05 is considered statistically significant.

drainage was reported to improve the outcome (*2,15,19*) and *Klebsiella* spp. was associated with a relatively benign course under aggressive medical care (*5,20*), we hypothesized that the decrease in death rates was due to the increase of *Klebsiella* spp. infection and the increase of abscess drainage. To test this hypothesis, we calculated the trends of death-related factors over time in the NHI database. As indicated in Table 8, the decrease in death rates was chronologically compatible with the increase of abscess drainage (+1.38% per year, r = 0.88, p = 0.002). Although the database did not include culture data, the annual increase of diabetes (+0.69%, r = 0.86, p = 0.003) and pneumonia (+0.28%, r = 0.90, p = 0.001) suggested the concomitant annual increase of *Klebsiella* spp. infection. Therefore, the significant decrease of liver abscess-related deaths in recent years is caused by a microbiologic shift (more *Klebsiella* spp. infections) and better medical care (more abscess drainage).

Our study suggests that both underlying renal disease and malignancy increased the incidence and mortality rates of pyogenic liver abscess. A previous survey in patients with end-stage renal disease showed the in-hospital prevalence of liver abscess to be 130.59/100,000 and the death rate to be 33.30% (*21*). Patients died of septic shock despite aggressive management in 50% of these cases. In our data, renal disease triples the incidence of pyogenic liver abscess and doubles the death rates, compatible with the previous report. Similarly, underlying malignancy had been well recognized as an important aggravating factor (*13,22*) of liver abscess. Our data also showed 10-fold and 2-fold increases in the incidence and mortality rates of pyogenic liver abscess. In addition to the fact that tumors from the hepatobil-

Table 7. Factors modifying the death rates from primary pyogenic liver abscess analyzed by binary logistic regression, NTUH, Taiwan, 2000–2004*

Factor	Univariate analysis			Multivariate analysis		
	Odds ratio	p value†	95% CI	Odds ratio	p value†	95% CI
Age	1.034	0.074	0.997–1.073	–	–	–
Gender	0.436	0.117	0.154–1.231	–	–	–
Klebsiella	0.201	0.003	0.070–0.574	0.223	0.009	0.072–0.690
Diabetes mellitus	1.088	0.880	0.363–3.260	–	–	–
Renal disease	4.256	0.019	1.269–14.271	6.172	0.006	1.665–22.884
Heart disease	1.354	0.698	0.293–6.247	–	–	–
Malignancy	4.433	0.016	1.319–14.900	2.922	0.119	0.759–11.254
Cirrhosis	4.012	0.212	0.452–35.613	–	–	–
Abscess drainage	0.345	0.045	0.122–0.977	0.351	0.063	0.116–1.060
Hospitalization days	1.002	0.793	0.990–1.013	–	–	–

*NTUH, National Taiwan University Hospital; CI, confidence interval.
†p<0.05 is considered statistically significant.

Table 8. Chronologic changes of factors associated with pyogenic liver abscess, NHI database, Taiwan, 1996–2004*

Year (no. cases)	Abscess drainage, no. (%)	Biliary procedure, no. (%)	Renal disease, no. (%)	Diabetes mellitus, no. (%)	Hepatobiliary malignancy, no. (%)	Other malignancy, no. (%)	Pneumonia, no. (%)	Heart disease, no. (%)	Deaths, no. (%)
1996 (2,400)	589 (24.5)	316 (13.2)	123 (5.1)	696 (29)	149 (6.2)	81 (3.4)	132 (5.5)	137 (5.7)	296 (12.3)
1997 (2,607)	729 (28)	389 (14.9)	156 (6)	819 (31.4)	210 (8.1)	106 (4.1)	155 (5.9)	154 (5.9)	308 (11.8)
1998 (1,831)	932 (32.9)	473 (16.7)	161 (5.7)	911 (32.2)	276 (9.7)	124 (4.4)	168 (5.9)	186 (6.6)	340 (12)
1999 (3,098)	1,055 (34.1)	480 (15.5)	198 (6.4)	1,005 (32.4)	302 (9.7)	137 (4.4)	179 (5.8)	194 (6.3)	351 (11.3)
2000 (3,394)	1,190 (35.1)	484 (14.3)	258 (7.6)	1,139 (33.6)	292 (8.6)	171 (5)	193 (5.7)	198 (5.8)	348 (10.3)
2001 (3,671)	1,233 (33.6)	515 (14)	286 (7.8)	1,241 (33.8)	340 (9.3)	203 (5.5)	246 (6.7)	254 (6.9)	418 (11.4)
2002 (3,891)	1,355 (34.8)	604 (15.5)	279 (7.2)	1,339 (34.4)	372 (9.6)	206 (5.3)	290 (7.5)	264 (6.8)	410 (10.5)
2003 (3,820)	1,387 (36.3)	519 (13.6)	281 (7.4)	1,411 (36.9)	347 (9.1)	195 (5.1)	282 (7.4)	290 (7.6)	381 (10.0)
2004 (3,991)	1,498 (37.5)	548 (13.7)	221 (5.5)	1,343 (33.7)	344 (8.6)	260 (6.5)	303 (7.6)	267 (6.7)	388 (9.7)
Change per y, %	+1.38	(–0.10)	+0.17	+0.69	+0.20	+0.31	+0.28	+0.17	(–0.31)
r	0.88	–0.23	0.46	0.86	0.49	0.93	0.90	0.75	–0.91
p value†	0.002	0.547	0.208	0.003	0.179	<0.001	0.001	0.020	0.001

*NHI, National Health Insurance; *r*, Pearson correlation coefficient.
†p<0.05 is considered statistically significant.

iary region might mimic abscesses (*23–25*), tumors in other regions were also associated with poor prognoses (*26,27*), confirming malignancy itself as an independent aggravating factor. Because the prevalence of both renal disease and malignancy has increased in recent years, the threat of pyogenic liver abscess in those patients is becoming more important, worthy of our special notice.

In contrast to renal disease and malignancy, the role of diabetes in mortality rates is controversial. Some reports suggested its association with a more aggressive clinical course (*18,28*) while others merely confirmed its coexistence with pyogenic liver abscess (*15,19,22*). In our analysis of the NHI database, diabetes caused a 9-fold increase of incidence but paradoxically decreased death rates. Further analysis in NTUH case-patients showed that such a decrease was attributed to the high proportion of *Klebsiella* spp. infection and that diabetes played no significant role in prognoses. This result is compatible with a report from a recent population-based case-control study in Denmark which stated that diabetes modified the risk but not the prognosis of pyogenic liver abscess (*29*). Since diabetes compromises host immune systems, its pivotal role in the risk for abscess but minimal role in abscess-related death, indicates a complicated interaction between the pathogens and the immune system of hosts. More in-depth research in this field is necessary.

The NHI database, although almost complete, is limited in 3 aspects. First, it is deficient in microbiologic data. Because the microbiologic data from NTUH is hospital-based, data might not accurately reflect the condition in the general population. Second, because it enables only 5 diagnoses for each case, coding of diagnoses might be biased if the specific case is complicated with >5 underlying diseases. For this reason, some minor conditions, such as peptic ulcer, urinary tract infection, and hypertension, paradoxically decreased death rates in our data. Third, in contrast to the comprehensive data of pyogenic liver abscess, detailed health data for each person in the population are not available. We are therefore unable to estimate the interaction among the risk factors of pyogenic liver abscess in the population (Table 3). Nevertheless, this study still provides a clear picture of pyogenic liver abscess in Taiwan. The rapid and steady increase of cases with pyogenic liver abscess in Taiwan should be noted (Table 2). Although the prognosis of liver abscess patients has improved over time (Figure 3), pyogenic liver abscess-related death in the population continues to increase (Table 2). Furthermore, complex interactions between pyogenic liver abscess, diabetes, renal disease, and malignancy are shown to worsen this condition. Further collaboration among clinical medical practitioners, public health workers, and research scientists is mandatory to fight against such a challenge in the future.

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Estimating Community Incidence of *Salmonella*, *Campylobacter*, and Shiga Toxin–producing *Escherichia coli* Infections, Australia

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To estimate multipliers linking surveillance of salmonellosis, campylobacteriosis, and Shiga toxin–producing *Escherichia coli* (STEC) infections to community incidence, we used data from a gastroenteritis survey and other sources. Multipliers for severe (bloody stool/long duration) and milder cases were estimated from the component probabilities of doctor visit, stool test, sensitivity of laboratory test, and reporting to surveillance system. Pathogens were classified by the same severity criteria and appropriate multipliers applied. Precision of estimates was quantified by using simulation techniques to construct 95% credible intervals (CrIs). The multiplier for salmonellosis was estimated at 7 (95% CrI 4–16), for campylobacteriosis at 10 (95% CrI 7–22), and for STEC at 8 (95% CrI 3–75). Australian annual community incidence rates per 100,000 population were estimated as 262 (95% CrI 150–624), 1,184 (95% CrI 756–2,670), and 23 (95% CrI 13–54), respectively. Estimation of multipliers allows assessment of the true effects of these diseases and better understanding of public health surveillance.

The primary aims of laboratory-based surveillance of *Salmonella, Campylobacter,* and Shiga toxin–producing *Escherichia coli* (STEC) infections in industrialized countries are to detect outbreaks and to monitor changes in incidence over time. For laboratory-based surveillance, a person with diarrhea must visit a doctor, have an appropriate stool sample transported to the laboratory correctly, have a positive laboratory test for a notifiable disease, and have this result reported to the surveillance system. Because many persons do not visit a doctor or have a stool sample taken when they have diarrhea, surveillance data do not capture all disease and represent only a fraction of disease occurring in the community. However, knowledge of the absolute number of cases in the community would be extremely useful for setting public health policy and estimating the cost of illness.

Studies that have estimated community incidence have used various methods. These include: capture-recapture (*1*), Delphi or expert consensus (*2*), outbreak reports (*3*,*4*), community-based cohort studies of diarrheal disease (*5*), and estimation of multipliers of surveillance data by using additional data (*6*). Although cohort studies are the most direct way of estimating diarrheal illness in the community, they are expensive and are subject to limitations for estimating the incidence of pathogen-specific infections because of the small numbers of cases (*7*,*8*).

This article describes how we estimated multipliers to apply to laboratory surveillance data to estimate community incidence, including estimation of precision. We used data collected in a survey of gastroenteritis and data from case-control studies and from quality assurance of laboratory testing to estimate the major component parts of the multipliers. The major components are the proportion of case-patients who visit a doctor, the proportion of these patients who have a stool sample sent to the laboratory for testing, the sensitivity of the laboratory test to correctly identify a pathogen, and the proportion of positive results that are reported to surveillance by laboratories. These component parts were then used to estimate the

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overall multipliers, and the precision of these estimates was determined.

In 2005, a total of 7,720 cases of salmonellosis, 15,313 cases of campylobacteriosis, and 85 cases of STEC infection were reported to the Australian National Notifiable Diseases Surveillance System. The specific objective of this study was to estimate the multipliers to apply to *Salmonella, Campylobacter,* and STEC infections reported to national surveillance and to estimate the community incidence of these conditions in Australia.

Methods

Overview

The fraction of community cases reported to surveillance was derived from the probability of a case-patient in the community visiting a doctor, having a stool sample taken, having a positive laboratory test, and having the case reported to surveillance (Figure). At every step a proportion of cases from the previous step are lost, resulting in only a fraction of cases being reported (the notification fraction).

The notification fraction (NF) is equal to the product of the component probabilities; that is, NF = $P_d \times P_s \times P_l \times P_n$ (Figure), where P_d = probability that a case-patient in the community visits a doctor, P_s = probability that a case-patient seen by a doctor has a stool sample taken, P_l = probability that a laboratory correctly identifies the pathogen in the stool sample, and P_n = probability that a positive result is reported to health authorities. The reciprocal of the notification fraction is the multiplier M (M = 1/NF).

The probabilities of visiting a doctor and of having stool tested are likely to be greater when illness is more severe.

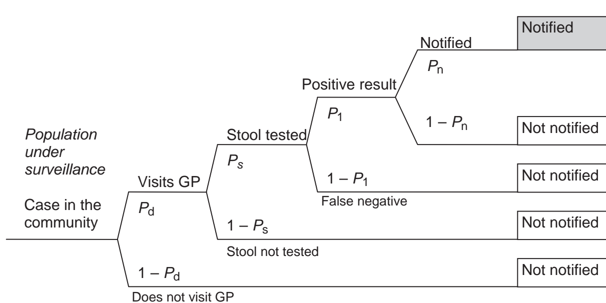

Figure. Sequential steps for notification to a surveillance system. The probability of progressing in the sequential steps in the surveillance system is represented by *P*. GP, general practitioner.

We therefore assessed the influence of different symptoms on the likelihood of gastroenteritis patients visiting a doctor and having a stool sample taken. Influential symptoms were used to classify gastroenteritis cases by severity, and multipliers were calculated for the different severity categories. The 3 infections of interest—salmonellosis, campylobacterosis, and STEC infection—were then classified by using the same categories so that appropriate multipliers could be applied, based on the severity of the particular illness. Uncertainty in the estimates was estimated by using simulation techniques to derive a 95% credible interval (95% CrI), akin to methods used in Bayesian estimations.

Data Sources

We derived our estimates from data already available in Australia on diarrhea, salmonellosis, campylobacteriosis, and STEC infection (Table 1).

Table 1. Data sources used to assess under-reporting of *Salmonella*, *Campylobacter*, and STEC infections, Australia, 2001–2005*

Information	Data sources
Symptoms that predicted visiting a doctor and having stool tested (“predictor symptoms”) used to adjust calculations for severity of illness	Australian National Gastroenteritis Survey (NGS) conducted across Australia during 2001 and 2002 (*9*)
Probability of a case-patient in the community visiting a doctor	NGS
Probability of a case-patient seen by a doctor having stool tested	NGS and unpublished reports of 2 surveys of GP treatment and management practices for gastroenteritis in 2003/2004 and 2005 in 2 Australian states (*10,11*)
Probability of correctly identifying *Salmonella* and *Campylobacter* in stool samples by laboratories	Royal College of Pathologists Australasia, Quality Assurance Programs Pty Limited, Microbiology QAP Results, 2001 (*12*)
Probability of a positive result being reported to health authorities	Discussions with OzFoodNet epidemiologists
Symptom profiles for reported cases of salmonellosis	Unpublished case-control study data from the Hunter Public Health Unit, NSW Australia (1997–2000), and OzFoodNet sites (2000–2003)
Symptom profiles on reported cases of campylobacteriosis	Unpublished case control study data from the Hunter Public Health Unit, NSW Australia (1997–2000), and OzFoodNet sites (2000–2003)
Information on reported cases of STEC, and laboratory sensitivity of detecting STEC from fecal samples	Unpublished data from OzFoodNet study on STEC in South Australia, 2003–2005
Number of notifications of campylobacteriosis, salmonellosis, and STEC infection.	National Notifiable Diseases Surveillance System (*13)*
Australian midyear population for 2005	Australian Bureau of Statistics (*14)*

*STEC, Shiga toxin–-producing *Escherichia coli;* GP, general practitioner; QAP, quality assurance program. Further details of how data were used are shown in the online Technical Appendix, available from www.cdc.gov/EID/content/14/10/1601-Techapp.pdf.

Estimation of Symptom-specific Probabilities for Visiting a Doctor and Having a Stool Test (P_d and P_s)

The component probabilities P_d and P_s were estimated from the National Gastroenteritis Survey (NGS) conducted in 2002. The telephone survey, using a random stratified sample from all states in Australia, included a total sample of 6,087 persons who were asked about diarrheal illness they had experienced in the previous 4 weeks. Respondents with chronic illness with diarrhea were not counted as case-patients unless they identified their symptoms as different than usual. Case-patients were asked details about their illness and days of duration of symptoms (*9*). The case definition of diarrheal illness was the following: at least 3 loose stools in 24 hours (excluding those persons who report a noninfectious cause of their diarrhea because of pregnancy, alcohol use, chronic illness, or medications) and duration <28 days.

Adjusting Component Probabilities for Severity of Illness

To calculate improved pathogen-specific estimates rather than estimates of underreporting of all gastroenteritis, we estimated multipliers from NGS for gastroenteritis of varying severity. The proportion of cases in corresponding severity categories was then estimated for the 3 different pathogens by using other data from case-control studies (Table 1). The appropriate multipliers could then be applied according to the average severity of illness.

Symptoms of severity that predicted whether the person with gastroenteritis visited a doctor were identified from NGS by using multivariable logistic regression. The following conditions were evaluated: duration of illness, cramps, vomiting, nausea, blood in the stool, headache, respiratory symptoms (coughing, sneezing, sore throat), fever, body aches, loss of appetite, and stiff neck. Age and sex of the patients were also considered. A p value <0.05 was considered statistically significant.

Because the number of case-patients who had stool tests was small in the gastroenteritis survey (n = 15), univariate regression was used to identify severity symptoms that predicted whether a doctor ordered a stool test (statistical significance $p<0.1$). Additional information from a random sample of general practitioners (GPs) from 2 states of Australia on the treatment of diarrheal disease was used to identify severity symptoms that prompted doctors to order a stool test (*10,11*). Data were stratified by symptom categories that were statistically significant determinants for both visiting a doctor and having a stool test ordered. Probabilities were calculated for case-patients in each symptom category of visiting a doctor and having stool tested.

Pathogen-specific Symptom Profiles

The severity symptom profiles of salmonellosis and campylobacterosis were developed from OzFoodNet national case-control studies based on 396 and 1,087 reported cases, respectively (Table 1). The STEC symptom profile was based on a case-control study of 34 cases in South Australia. The proportion of case-patients who experienced the predictor symptoms (i.e., symptoms that were found to predict visiting a doctor and a doctor's ordering of stool tests) was calculated. From this calculation, the probabilities of visiting a doctor and having a stool test were estimated for each pathogen, weighted by severity of illness.

Sensitivity of Laboratory Testing

All fecal specimens are routinely tested for *Salmonella* and *Campylobacter* spp. in Australia. The sensitivities (P_l) of laboratory tests to detect *Salmonella* and *Campylobacter* spp. were obtained from quality assurance data from ≈250 laboratories across the country (*12*). This sensitivity testing is based on inoculated samples that are transported to laboratories from a central source. For STEC, data were from the state of South Australia, where enhanced surveillance exists for this pathogen. Laboratories routinely forward all stool samples with macroscopic blood to a reference laboratory, which tests for STEC by using molecular methods to detect genes encoding for production of Shiga toxin 1 and Shiga toxin 2 (*15*).

Reporting to Surveillance Network

The completeness of mandatory reporting to health departments was discussed with state epidemiologists involved in OzFoodNet, Australia's foodborne disease surveillance network. Based on these discussions, we concluded that mandatory reporting to health departments has been virtually complete for these pathogens for several years preceding 2005 because of the widespread use of computerized reporting practices in Australian laboratories. This information was used to estimate the probability for a positive result to be reported to health authorities (P_n). Formal validation studies on completeness of reporting were not available.

Uncertainty and 95% CrIs

Simulation techniques were used to quantify the degree of uncertainty of the underreporting factors. The uncertainty in our knowledge about each of P_d, P_s, and P_l was quantified in terms of a normal distribution as this is a simple technique easily applied in different situations. However, because some data may be best simulated by using other distributions (such as probabilities close to 1 or 0 and other nonsymmetric distributions), we compared the output from simulations that used normal distributions with sim-

ulations that used other distributions as indicated (online Technical Appendix, available from www.cdc.gov/EID/content/14/10/1601-Techapp.pdf). The different distributions had minimal influence on the outcomes, and therefore all simulations were conducted as normal distributions.

The mean and standard deviation were allocated to describe the simulations when various data sources were used. The mean and standard deviation of the probability of visiting a doctor (P_d) were estimated from the gastroenteritis survey data (*9*). The mean and standard deviation of the probability of stool ordering (P_s) by GPs were quantified by averaging results from the GP surveys conducted in the states of South Australia and Victoria (*10,11*). The mean and standard deviation of the sensitivity of stool testing (P_l) were quantified by using quality assurance results from the 2001 report (*12*). If a simulation produced a few negative values, these were treated as missing data. For each pathogen, a distribution was similarly determined for the symptom profiles that described the proportion of cases in each of 6 severity categories by using data from the case-control studies as shown in Table 1.

For each pathogen, 1,000 observations were simulated from each of the distributions and were used to calculate 1,000 estimates of the multipliers. The range between the 2.5 and 97.5 percentiles of this empirical distribution was quoted as a 95% CrI for the final estimates of the multipliers. The parameters used in the simulations are shown in the online Technical Appendix tables. The software package SPSS was used for simulations (*16*).

Calculation of Community Incidence

The number of national notifications for each pathogen from 2000 through 2004 was used to estimate the mean and standard deviation of a normal distribution for the yearly notifications (Table 2). Data were adjusted for total population coverage for *Campylobacter* spp. because 1 state (New South Wales) does not report this pathogen. STEC estimates were based on information from 1 state (South Australia), so South Australian estimates for STEC were then extrapolated to the national population.

One thousand simulated values from these distributions were then multiplied by the 1,000 simulated values of the pathogen-specific multipliers to obtain 1,000 simulated annual incidences in the community. The median and the 95% CrI from this empirical distribution were estimated as above.

Results

Estimation of Symptom-specific Probabilities for Visiting a Doctor and Having a Stool Test

Among respondents of the NGS, 374 persons had diarrhea and met our case definition. Of these case-patients, 75 (20%) visited a doctor for treatment, and for 15 (20%) of 75, a stool test was ordered.

Duration of illness was the most important statistically significant symptom that increased both the probability of visiting a doctor and of having a stool sample taken. The odds of visiting a doctor doubled with each day of duration ($p<0.001$), and the odds of having a stool test among those who visited a doctor was 1.5 ($p = 0.005$) for each day of duration. Blood in stool was identified as a strong predictor for a doctor to order a stool test in both of the GP surveys, with 80% and 91%, respectively, of GPs in the 2 surveys "always or nearly always" ordering a stool test if blood was present (*10*,*11*). In the gastroenteritis survey, the presence of blood in the stool did not influence whether a person with gastroenteritis visited a doctor (odds ratio [OR] = 1.5, $p = 0.55$) but did influence whether a doctor ordered a stool test (OR = 9, $p = 0.08$). Fever was also statistically significantly associated with visiting a doctor. All other variables were not significant.

Since duration of illness was identified as influential on both the patient's visiting a doctor and on whether a stool test was ordered, and because blood in stool was also very influential on whether a doctor ordered a stool test, 6 severity categories were created: 3 duration categories (1–2 days, 3–4 days, ≥5 days) for those with blood in stool, and the same duration categories for those without blood in stool. The component probabilities P_d and P_s were then calculated separately for each severity category as shown in Table 3. Further data details are shown in the online Technical Appendix.

Because blood in stool did not predict patient's health-seeking behavior in the NGS, the estimates of probability of visiting a doctor by duration were made on the whole sample and then the same probabilities were applied to the categories of "with blood" and "without blood" in stool. The probability of visiting a doctor increased from 0.1 to 0.67 as duration increased from 1–2 days to ≥5 days. Based on the surveys of GPs, we estimated that among patients with bloody diarrhea who visited a doctor, the probability of having a stool test was 0.85, regardless of the duration of their diarrhea. For those without blood in stool, the probability of a stool test increased from ≈0.1 to 0.4 as duration of illness increased.

Laboratory Testing

Quality assurance testing in 2001 showed that 100% of 254 laboratories correctly detected *Salmonella* spp. from 1 batch of fecal samples, and 233 (95%) of 246 laboratories identified it correctly from a second batch. Of 257 laboratories, 220 (86%) correctly detected *Campylobacter* spp.from 1 batch of fecal samples, and 232 (95%) of 244 laboratories detected it from a second batch (*12*). The probability for correct identification of *Salmonella* spp. by laboratories

Table 2. Number of notifications in Australia each year for salmonellosis, campylobacteriosis, and STEC infections, 2000–2004†

Data	*Salmonella* infections	*Campylobacter* infections (all states except NSW)†	STEC infections in SA‡
Year			
2000	6,196	13,665	–
2001	7,047	16,123	27
2002	7,696	14,740	39
2003	7,017	15,369	37
2004	7,829	15,622	30
Mean (SD)	7,157 (651)	15,104 (946)	33.3 (5.67)
Median	7,047	15,369	34
Percentiles: 2.5, 97.5	6,278, 7,816	13,773, 16,073	27, 39

*STEC, Shiga toxin–producing *Escherichia coli*; NSW, New South Wales; SA, South Australia.
†67% of population only; adjust for population of Australia by multiplying by 1.5.
‡7.5% of population only; adjust for population of Australia by multiplying by 13.3.

was therefore estimated to be 0.98 (95% CrI 0.95–1.00) and for identification of *Campylobacter* spp., 0.90 (95% CrI 0.85–0.95).

The probability for correct STEC identification by a laboratory PCR was estimated to be 0.98 (95% CrI 0.95–1.00) (*17*). Macroscopic blood is the major reason for conducting a laboratory test to identity STEC in South Australia. Among those persons with STEC infection, 0.90 (95% CrI 0.85–0.95) of their stools are estimated to have macroscopic blood (*18*). The proportion of stools containing STEC that are identified by laboratories in South Australia was therefore estimated as the product of 0.90 (95% CrI; 0.85–0.95) and 0.98 (95% CrI 0.95–1.00) to give 0.88 (95% CrI; 0.83–0.93).

Pathogen-specific Multipliers

Underreporting varied considerably by severity of illness, with reporting ranging from ≈l in 2 cases for "severe illness" cases with blood in the stool and long duration, to ≈1 in 150 for "mild illnesses" without blood and shorter duration (Table 3). For every 100 cases reported, the number in each of the 6 severity categories is shown for each pathogen in Table 3. Long duration is common; 83% of salmonellosis cases, 77% of campylobacterosis cases, and 74% of STEC cases lasted at least 5 days. Blood in stool was reported for 50%, 44%, and 86% of salmonellosis, campylobacteriosis, and STEC cases, respectively. The severity-weighted estimates of underreporting for salmonellosis, campylobacteriosis, and STEC infection are shown in Table 4. For every 100 notifications, an estimated 695 (95% CrI 399–1,643) cases of salmonellosis occurred in the community and 1,001 (95% CrI 664–2,251) cases of campylobacteriosis. In South Australia, for every 100 notifications of STEC, an estimated 815 cases (95% CrI 330–7,514) occurred in the community.

The multipliers for *Salmonella, Campylobacter,* and STEC infections are thus 7 (95% CrI 4–16), 10 (95% CrI 7–22), and 8 (95% CrI 3–75), respectively. This indicates that overall, including mild to severe illness, ≈85% of salmonellosis cases, 90% of campylobacteriosis cases, and 88% of STEC cases are not reported.

Community Incidence of *Salmonella, Campylobacter,* and STEC Infections

In the 5 years from 2000 to 2004, the national notifications for *Salmonella* ranged from 6,196 to 7,829 each year. The notifications from all states, except New South Wales, for *Campylobacter* ranged from 13,665 to 15,622. The notifications for STEC in the state of South Australia ranged from 27 to 39 from 2001 through 2004 (Table 2). The number of yearly reported cases was used to estimate the mean and standard deviation and then data were simulated from a normal distribution to give distributions of average "yearly notification number." The product of the yearly notification number and the pathogen multipliers resulted in an estimate of the number of annual community infections of 49,843 (95% CrI 28,466–118,518) cases of salmonellosis, 224,972 (95% CrI 143,771–507,334) cases of campylobacteriosis, and 4,420 (95% CrI 2,407–10,196) cases of STEC infection. The corresponding estimates of annual incidence per 100,000 population are salmonellosis, 262 (95% CrI, 150–624); campylobacteriosis, 1,184 (95% CrI 756–2,670); and STEC infections, 23 (95% CrI 13–54).

Discussion

In this study, we were able to provide CrIs of annual community incidence of 3 important infections from surveillance data. We used a method for determining pathogen-specific underreporting factors in Australia that has been deduced without the need to collect costly new data and includes an estimation of precision. This method is applicable to diseases other than infectious diarrhea, provided data on the components of the notification fraction are available: the proportion of case-patients who visit a doctor, the proportion who have a laboratory test, the sensitivity of the test, and the completeness of reporting of illness to surveillance. Even if collecting some additional data is necessary to estimate certain components of the notification fraction, this collecting may be worthwhile to obtain the added insight into the effects of particular diseases. Although knowing the incidence may not be necessary for detecting outbreaks and monitoring increased

Table 3. Probabilities and underreporting factors for each category of duration of diarrhea by blood in stool, for salmonellosis, campylobacteriosis, and STEC infections*

Condition/predictor symptoms	Probability of: (a) Case-patient visiting a doctor (95% CrI)	(b) Stool being tested (95% CrI)	(c) Positive stool results (95% CrI)	(d) Notification by laboratory	Probability for a case to be reported† (95% CrI)	Multiplier‡ (95% CrI)
Salmonellosis						
With blood						
1–2 d	0.10 (0.07–0.14)	0.85 (0.72–0.98)	0.98 (0.95–1.00)	1.00	0.09 (0.06–0.12)	11.39 (8.49–16.36)
3–4 d	0.43 (0.31–0.54)	0.85 (0.72–0.98)	0.98 (0.95–1.00)	1.00	0.36 (0.25–0.46)	2.82 (2.17–3.98)
≥5 d	0.67 (0.46–0.88)	0.85 (0.72–0.98)	0.98 (0.95–1.00)	1.00	0.55 (0.368–0.75)	1.81 (1.33–2.72)
Without blood						
1–2 d	0.10 (0.07–0.14)	0.07 (0.02–0.02)	0.98 (0.95–1.00)	1.00	0.01 (0.003–0.01)	143.29 (83.30–371.0)
3–4 d	0.43 (0.31–0.54)	0.19 (0.071–0.36)	0.98 (0.95–1.00)	1.00	0.08 (0.010–0.16)	13.06 (6.37–67.83)
≥5 d	0.67 (0.46–0.88)	0.40(0.133–0.67)	0.98 (0.95–1.00)	1.00	0.25 (0.075–0.48)	3.93 (2.10–11.92)
Campylobacteriosis						
With blood						
1–2 d	0.10 (0.07–0.14)	0.85 (0.72–0.98)	0.90 (0.85–0.95)	1.00	0.08 (0.056–0.11)	12.40 (9.16–17.82)
3–4 d	0.43 (0.31–0.54)	0.85 (0.72–0.98)	0.90 (0.85–0.95)	1.00	0.33 (0.231–0.43)	3.06 (2.32–4.33)
≥5 d	0.67 (0.46–0.88)	0.85 (0.72–0.98)	0.90 (0.85–0.95)	1.00	0.51 (0.339– 0.70)	1.97 (1.42–2.95)
Without blood						
1–2 d	0.10 (0.07–0.14)	0.069 (0.02– 0.12)	0.90 (0.85–0.95)	1.00	0.01 (0.002–0.01)	154.17 (89.31–397.59)
3–4 d	0.43 (0.31–0.54)	0.185 (0.071–0.36)	0.90 (0.85–0.95)	1.00	0.07 (0.009–0.15)	14.15 (6.80–73.32)
≥5 d	0.67 (0.46–0.88)	0.400 (0.133–0.67)	0.90 (0.85–0.95)	1.00	0.24 (0.068–0.44)	4.25 (2.25–13.36)
STEC in South Australia						
With blood						
1–2 d	0.10 (0.07–0.14)	0.85 (0.72–0.98)	0.88 (0.83–0.93)	1.00	0.08 (0.0.05–0.11)	13.02 (9.50–18.37)
3–4 d	0.43 (0.31–0.54)	0.85 (0.72–0.98)	0.88 (0.83–0.93)	1.00	0.32 (0.22–0.42)	3.13 (2.36–4.45)
≥5 d	0.67 (0.46–0.88)	0.85 (0.72–0.98)	0.88 (0.83–0.93)	1.00	0.50 (0.33–0.68)	2.02 (1.47–3.04)
Without blood						
1–2 d	0.10 (0.07–0.14)	0.07 (0.02–0.12)	0.88 (0.83–0.93)	1.00	0.01 (0.001–0.02)	157.18 (61.67–218.75)
3–4 d	0.43 (0.31–0.54)	0.19 (0.071–0.36)	0.88 (0.83–0.93)	1.00	0.07 (0.01–0.14)	14.35 (7.38–64.34)
≥5 d	0.67 (0.46–0.88)	0.40 (0.133–0.67)	0.88 (0.83–0.93)	1.00	0.23 (0.07–0.44)	4.31 (2.27–13.44)

*STEC, Shiga toxin–producing *Esherichia coli*; CrI, credible interval; NF, notification factor.
†NF, product of a × b × c × d.
‡Inverse of NF.

or decreased disease over time, this information is vitally important to policy makers. We consider it most important to furnish estimates with a measure of their precision and have used a simple simulation technique that is easily applied. If information is to be used in public health policy making, the responsible interpretation of results involves a realistic appreciation of potential error. Simple point estimates may give a misleading picture of certainty; the estimates of the community incidence of these foodborne diseases show a high degree of uncertainty that should be acknowledged when comparing estimates from other countries or times.

If this level of uncertainty is also found in other countries, then our confidence in apparent differences may be

Table 4. Severity-specific underreporting for salmonellosis, campylobacteriosis, and STEC infections*

Condition/severity category	Symptom multiplier† (95% CrI)	No. reported cases in severity category, in hundreds (95% CrI)	No. cases in the community for every 100 reported‡ (95% CrI)
Salmonellosis			
With blood			
1–2 d	11.39 (8.49–16.36)	1 (0–3)	12.7 (0.8–32.1)
3–4 d	2.82 (2.17–3.98)	7 (5–10)	19.9 (12.6–30.9)
≥ 5 d	1.81 (1.33–2.72)	42 (37–47)	76.6 (54.3–116.0)
Without blood			
1–2 d	143.29 (83.30–371.0)	2 (1–4)	282.6 (50.4–870.3)
3–4 d	13.06 (6.37–67.83)	7 (5–10)	91.8 (40.3–533.5)
≥5 d	3.93 (2.10–11.92)	41 (36–46)	160.8 (85.8–513.8)
Overall		100	695 (399–1,643)
Campylobacteriosis			
With blood			
1–2 d	12.40 (9.16–17.82)	2(1–3)	24.8 (16.3–38.6)
3–4 d	3.06 (2.32–4.33)	8(6, 10)	24.3 (15.8–36.9)
≥5 d	1.97 (1.42–2.95)	34 (31, 37)	67.92 (48.5–106.3)
Without blood			
1–2 d	154.17 (89.31–397.59)	3 (2–4)	475.7 (250.6–1,234.3)
3–4 d	14.15 (6.80–73.32)	10 (8–12)	139.0 (68.7–739.7)
≥5 d	4.25 (2.25–13.36)	43 (40–46)	183.4 (97.9–578.1)
Overall		100	1001 (664–2,251)
STEC in South Australia			
With blood			
1–2 d	13.02 (9.50–18.37)	0	0
3–4 d	3.13 (2.36–4.45)	18 (6–30)	51.6 (16.2–101.2)
≥5 d	2.02 (1.47–3.04)	68 (50–87)	123.5 (74.9–212.9)
Without blood			
1–2 d	157.18 (61.67–218.75)	3 (1–5)	432.5 (142.4–1,220.1)
3–4 d	14.35 (7.38–64.34)	6(1–11)	78.0 (13.6–400.1)
≥5 d	4.31 (2.27–13.44)	6 (1–11)	2-3.0 (2.67–91.7)
Overall	13.02 (9.50–18.37)	100	815 (330–7,514)

*STEC, Shiga toxin–producing *Esherichia coli*.
†Number of cases in the community for every notification.
‡Product of previous 2 columns.

compromised. However, some differences appear to be so large that they are of interest nonetheless. When compared with multipliers for enteric diseases in other countries, Australia's estimates were most similar to the estimates in the United Kingdom that were derived from a cohort study. It was estimated that for every case reported to surveillance, 3.2 cases of salmonellosis and 7.6 cases of campylobacteriosis existed in the community (*5*). However, the multiplier for salmonellosis in the United States has been estimated in the past at 39 (*3*,*19*), and the same factor was estimated in a recent US study (*6*). This recent study estimated the component probabilities of the notification fraction by using data from a population survey of diarrhea from 1996 in which the proportion of case-patients who visited a doctor was 12%. More recent surveys in the United States have put this estimate at ≈20% (*20*). If 20% is now more appropriate, then the US multiplier would reduce to ≈25. In the US study, blood in stool was found to be highly influential on stool test ordering by doctors (100% vs. 18% requested stool tests, depending on blood in stool) and not so influential on the probability of a case-patient visiting a doctor (15% vs. 12%), which was similar to our Australian study findings. The US study did not report duration as a predictor of visiting a doctor and of having a stool test. Because salmonellosis frequently lasts >5 days, adjusting for duration had a marked impact on our multiplier, reducing it considerably. If a similar influence of duration on visiting a doctor and ordering stool tests exists in the United States, and the symptom profile of salmonellosis is similar in the 2 countries, then the US multiplier would likely be further reduced.

The choice of the case definition of diarrheal illness used in population studies may also have affected calculations for the multipliers when the method of component probabilities was used. If the case definition itself includes features of severity that are predictors of visiting a doctor or of having a stool test, this may affect the proportion of cases that undergo these steps, thereby affecting the component probabilities used to calculate the multiplier. The laboratory sensitivity testing that provided another com-

ponent probability may also affect the calculation of the multipliers. The quality assurance testing mimics some of the transport issues that occur in real life, but it probably represent a "best cases scenario" in which a patient sheds microorganisms at the time of collection, and good transport methods are available.

In addition to unavoidable uncertainty due to paucity of data, methodologic differences in each study, combined with differences in surveillance systems, can make international comparisons of disease incidence difficult. However, applying the multiplier for each country leads to an estimate of community incidence that is likely to make comparisons more meaningful than simply comparing notification rates. The notification rates of salmonellosis in each country are currently ≈70/100,000 population for the United Kingdom (*5*), 38/100,000 for Australia, and 12/100,000 (*21*) for the United States. Applying the underreporting factors of 3, 39, and 7 for the United Kingdom, United States, and Australia gives estimates of annual community incidence of ≈220/100,000, ≈470/100,000, and ≈262/100,000 (95% CrI 150–624), respectively. If more recent estimates of the proportion of case-patients who visit a doctor are used to calculate the underreporting factor in the United States, and a factor of 25 were applied to the number of notifications, the US rate becomes 300/100,000.

To validate results or assess the potential degree of error, another useful approach is to use differing methods and compare results in the same country. In Australia, 1 other possible method is to use results from an Australian cohort study of diarrheal disease (*22*). The study was a randomized controlled trial, conducted for 18 months, that assessed the health impact of water quality and treatment in Melbourne, Victoria, in 1999. Of 795 stool samples tested, 9 cases of salmonellosis (0.003 per person-year) and 24 cases of campylobacteriosis (0.007 per person-year) were identified. When these data are extrapolated to the notifications in Victoria, the community-to-notification ratios are 12.6 for salmonellosis and 9.3 for campylobacteriosis, which are comparable to our estimated multipliers of 7 (95% CrI 4–16) for salmonellosis and 10 (95% CrI 7–22) for campylobacteriosis.

Assessment of the functionality of surveillance is likely to lead to more effective control of disease in the community, and multipliers are 1 measure of the quality of surveillance systems. The relatively low ratio between reported enteric cases and the number of community cases in Australia suggests that the surveillance system is working reasonably effectively and therefore is likely to detect outbreaks.

This study provides an estimate of the community incidence of 3 important foodborne diseases in Australia. Such estimates are important in public health to assess the economic and human cost of these diseases and to help set priorities. The estimates in this study show that salmonellosis, campylobacteriosis, and STEC have considerable effects in the community, most of which go unreported. Calculation of multipliers for other diseases would also be worthwhile to inform public health practice.

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Automatic Outbreak Detection Algorithm versus Electronic Reporting System

Masja Straetemans,[1] Doris Altmann, Tim Eckmanns, and Gérard Krause

To determine efficacy of automatic outbreak detection algorithms (AODAs), we analyzed 3,582 AODA signals and 4,427 reports of outbreaks caused by *Campylobacter* spp. or norovirus during 2005–2006 in Germany. Local health departments reported local outbreaks with higher sensitivity and positive predictive value than did AODAs.

In 2001, the Robert Koch Institute, Germany's federal institute for infectious disease control, implemented an electronic system (SurvNet) for notifiable infectious disease surveillance (*1*,*2*). Local health departments electronically sent reports of confirmed cases to state health departments, which forwarded them to Robert Koch Institute. SurvNet can link single case reports to outbreak reports in which local health departments report descriptive outbreak information in a standardized manner (reported outbreaks). Additionally, the same software organizes the electronic transmission of single case reports from peripheral databases from each local health department to databases of the respective state health department and finally to Robert Koch Institute. Automatic outbreak detection algorithms (AODAs), run weekly on this case-based data, generate signals when the observed number of cases per a specific week is higher than a defined threshold value (signal outbreaks).

To identify the need to follow up generated signals, one must know the positive predictive value of AODA. This knowledge could avoid overwork in local health departments because not every signal will require contacting the local office for investigation.

Our goal was to assess the probability that a signal generated by AODA reflects a real outbreak (*Campylobacter* spp. or norovirus) being reported by local health department. Previous studies have tested AODAs by comparing generated signals with simulated outbreaks superimposed on authentic syndromic surveillance data (*3*,*4*) or with a limited number of known natural outbreaks (*5*). In contrast to these approaches, we evaluated performance of AODA by comparing it with a large database of outbreaks electronically reported by local health departments, which we considered to be the reference standard (*2*).

The Study

We considered a signal outbreak to be identical to a reported outbreak when 1) ≥1 signal was triggered within the same period as the first and last case belonging to the particular reported outbreak, 2) the signal outbreak was associated with the identical geographic location on the municipal level (1 of the 430 municipalities) as the reported outbreak, and 3) the signal outbreak was associated with the identical pathogen (either *Campylobacter* spp. or norovirus). Using the data available as of June 1, 2007, we considered the number of reported outbreaks (a minimum of 4 cases because the algorithm cannot detect outbreaks with <4 cases), from week 5 of 2005 through week 4 of 2007.

During the study period, 118 and 4,309 outbreaks with ≥4 cases, associated with the pathogens *Campylobacter* spp. and norovirus, respectively, had been reported. The AODA had signaled 52 (44.1%) of the 118 reported *Campylobacter* spp. outbreaks and 2,538 (58.9%) of the 4,309 reported norovirus outbreaks (Table). The probability that a signal outbreak reflected a reported outbreak (positive predictive value of AODA) was lower for *Campylobacter* spp. than for norovirus: 50 (6.4%) of 781 *Campylobacter* spp. signal outbreaks and 2,115 (75.5%) of 2,801 norovirus signal outbreaks were associated with reported outbreaks. The AODA may have triggered multiple signals during the outbreak if the threshold level was reached during several consecutive weeks (Figure 1). Of the *Campylobacter* spp. outbreaks, 3 (6.0%) were each identified by 2 different signals; of the norovirus outbreaks, 727 (28.6%) were identified by multiple signals (2–20 signals per reported outbreak) (Table). Furthermore, 1 signal outbreak could correspond with different reported outbreaks when these occurred in the same local area and during the same period (Figure 2). For *Campylobacter* spp., 4 (8.0%) of the outbreak signals could correspond to >1 reported outbreak; for norovirus, 760 (35.9%) of the signal outbreaks could correspond to 2–26 reported outbreaks (Table).

Conclusions

Germany´s electronic reporting system for infectious disease outbreaks provided a unique opportunity to compare the triggering of signals through AODA with the reporting of outbreaks identified by local health departments. The probability of an outbreak signal being associated with a reported outbreak was much lower for *Campylobacter* spp. (6.4%) than for norovirus (75.5%). Furthermore, the fraction of cases as part of a reported outbreak was much lower for *Campylobacter* spp. (3.3%) than for norovirus

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Table. Outbreaks January 31, 2005–January 28, 2007, reported and identified by detection algorithm*

Outbreak characteristic	*Campylobacter* spp., no. (%)	Norovirus, no. (%)
Total cases	114,176	144,568
Cases as part of a reported outbreak	3,767 (3.3)	103,177 (71.4)
Reported outbreaks with <4 cases	1,453	5,074
Reported outbreaks with ≥4 cases	118	4,309†
Signal outbreaks generated by detection algorithm	781	2,801
Reported outbreaks with ≥4 cases identified by detection algorithm signals	52 (100)	2,538 (100)
Reported outbreaks identified by 1 signal	49 (94.0)	1,811 (71.4)
Reported outbreaks identified by >1 signal	3 (6.0)	727 (28.6)
Reported outbreaks identified by 2 signals‡	3 (6.0)	473 (18.6)
Reported outbreaks identified by >2 signals‡	0	254 (10.0)
Signal outbreaks corresponding to reported outbreak with ≥4 cases	50 (100)	2,115 (100)
Signal outbreaks corresponding to 1 reported outbreak	46 (92.0)	1,355 (64.1)
Signal outbreaks corresponding to >1 reported outbreak	4 (8.0)	760 (35.9)
Signal outbreaks corresponding to 2 reported outbreaks§	3 (6.0)	408 (19.3)
Signal outbreaks corresponding to >2 reported outbreaks§	1 (2.0)	352 (16.7)

*Data through June 1, 2007. Sensitivity detection algorithm 44.1% (52/118) for *Campylobacter* spp., 58.9% (2,538/4,309) for norovirus; no. reported outbreaks with ≥4 cases also identified by detection algorithm signal/total no. reported outbreaks with ≥4 cases. Positive predictive value of detection algorithm 6.4% (50/781) for *Campylobacter* spp.*,* 75.5 (2,115/2,801) for norovirus. No. signal outbreaks identical to reported outbreak/total number of signal outbreaks.
†Excluded are 17 reported norovirus outbreaks of >25 wk and an average of <2 cases/wk because these are likely the result of data entry errors in SurvNet.
‡During the duration of a reported outbreak, the detection algorithm may have triggered multiple signals during several consecutive weeks (Figure 1).
§One signal outbreak may correspond to multiple reported outbreaks if different outbreaks occur in the same municipality during the same period (Figure 2).

(71.4%). Differences in route of transmission likely explain why *Campylobacter* spp. cases are generally more likely to occur sporadically and why norovirus cases are more likely to be part of an outbreak (*6–9*). These differences might result in a lower frequency of *Campylobacter* spp. outbreaks. The AODA might generate a signal when a higher than expected number of single cases is observed in a specific period and location, but this signal is likely to reflect an increased number of sporadic cases; an increased number of norovirus cases is more likely to reflect an occurring norovirus outbreak. An alternative possibility is that local health departments are more inclined to identify, investigate, and report norovirus outbreaks than *Campylobacter* spp. outbreaks (*10*). These differences demonstrate the importance of designing AODA specifically for the pathogens under surveillance.

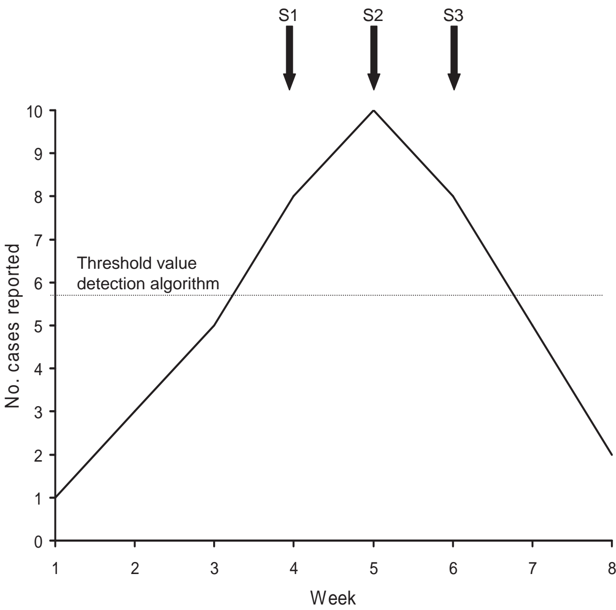

Figure 1. Example of 1 reported outbreak being detected by 3 signals. In this example, 3 signal outbreaks (S1, S2, S3) can be associated with 1 reported outbreak in same municipality and during the same period.

For our analyses we used reported outbreaks as the reference standard by which to evaluate the AODA. Although this outbreak reporting is probably incomplete, we believe that it more closely identifies the true number of outbreaks than does retrospectively identifying outbreaks (*11*) or simulating outbreaks (*3*,*4*). Thus, we believe it generates a better reference standard than that used in previous studies.

Our findings question the usefulness of the AODA because a large number of generated signals were not confirmed by the electronic outbreak reporting from local health departments. Our results suggest that AODAs are not useful for detecting outbreaks on a local level because the outbreaks are detected earlier and investigated by the local health department. AODAs might be more useful for detecting multicounty or even multistate outbreaks, which are more difficult to detect by a single local health department. The latter has been well demonstrated by AODA detection of various foodborne outbreaks in Germany (*12*,*13*). National surveillance should focus on the follow-up of signals that indicate potential multicounty or multistate outbreaks. We used the county level for the algorithm because we obtain the reported outbreaks on this level first and we wanted to compare both systems. Our standard algorithms run also

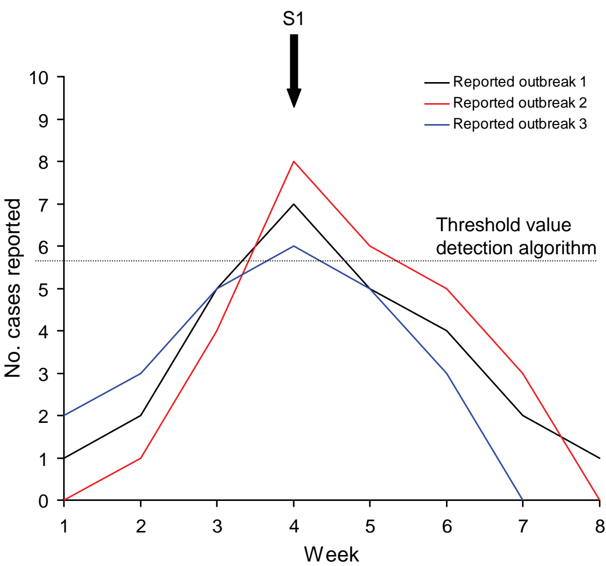

Figure 2. Example of 1 signal outbreak corresponding to multiple reported outbreaks. In this example, 1 signal outbreak (S1) can be associated with 3 reported outbreaks occuring in same municipality; threshold is reached in same week number.

on federal and state levels, but that was not the subject of this investigation. To enable local health departments to earlier discover multicounty outbreaks, a new version of SurvNet is being developed. This version will give local health departments the opportunity to include more information on the evidence and also the possibility of linking outbreaks from different counties (*2*). The Robert Koch Institute, along with the state health departments, will develop a standard operating procedure for how to communicate and follow up on signals generated by the AODA.

Our study suggests that the usefulness of AODA to detect local outbreaks is limited because local health departments generally detect local outbreaks earlier and in more detail than these algorithms. Investment in the development of user-friendly outbreak reporting tools for local health departments might therefore provide better information on outbreaks than extensive refinements of AODAs.

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Rickettsia typhi and *R. felis* in Rat Fleas (*Xenopsylla cheopis*), Oahu, Hawaii

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Rickettsia typhi (prevalence 1.9%) and *R. felis* (prevalence 24.8%) DNA were detected in rat fleas (*Xenopsylla cheopis*) collected from mice on Oahu Island, Hawaii. The low prevalence of *R. typhi* on Oahu suggests that *R. felis* may be a more common cause of rickettsiosis than *R. typhi* in Hawaii.

Murine typhus is a febrile zoonotic disease caused by *Rickettsia typhi*. The classic view is that *R. typhi* circulates among rats (*Rattus rattus* or *R. norvegicus*) and rat fleas (*Xenopsylla cheopis*) (*1*,*2*), although other rodents and their ectoparasites have been implicated in maintenance of *R. typhi* in nature. Humans become infected when they visit disease-endemic areas infested with rats and acquire infection by inhalation or by self-inoculating infected fleas or flea feces into skin.

The most recent outbreak of murine typhus in Hawaii occurred in 2002 with 47 cases reported on 5 islands (*3*). Concomitantly, an increase occurred in the mouse population on the island of Maui, which reported 35 human cases. Peak occurrence of murine typhus in Hawaii was in 1944 with 186 cases reported, of which 80% occurred on the island of Oahu (*4*). Previous serologic surveys in Hawaii have identified antibodies reactive with *R. typhi* antigen in the Polynesian rat (*R. exulans*), black rat (*R. rattus*), Norway rat (*R. norvegicus*), and house mouse (*Mus musculus*) (*3,4*). The Indian mongoose (*Herpestes auropunctatus*) was also identified as a potential reservoir; however, its role has not yet been evaluated. We conducted a molecular survey of fleas in Oahu to characterize the prevalence and identity of rickettsiae in Hawaii.

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The Study

M. *musculus* mice were collected during rodent population studies in the leeward and southeast parts of Oahu during the summers of 2004, 2006, and 2007 (Figure). Fleas were combed from each animal, identified as *X*. *cheopis* by using standard taxonomic keys, and kept frozen at –70°C until they were sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for further analyses. Mice were humanely killed; only brains were removed and frozen.

DNA was isolated from each flea by using the Biomek 2000 Laboratory Automation workstation (Beckman, Fullerton, CA, USA) and reagents from the Wizard Prep kit (Promega, Madison, WI, USA) (*5*). DNA from 20 mg of mouse brain tissue was isolated by using the QiaAmp Mini kit (QIAGEN, Valencia, CA, USA).

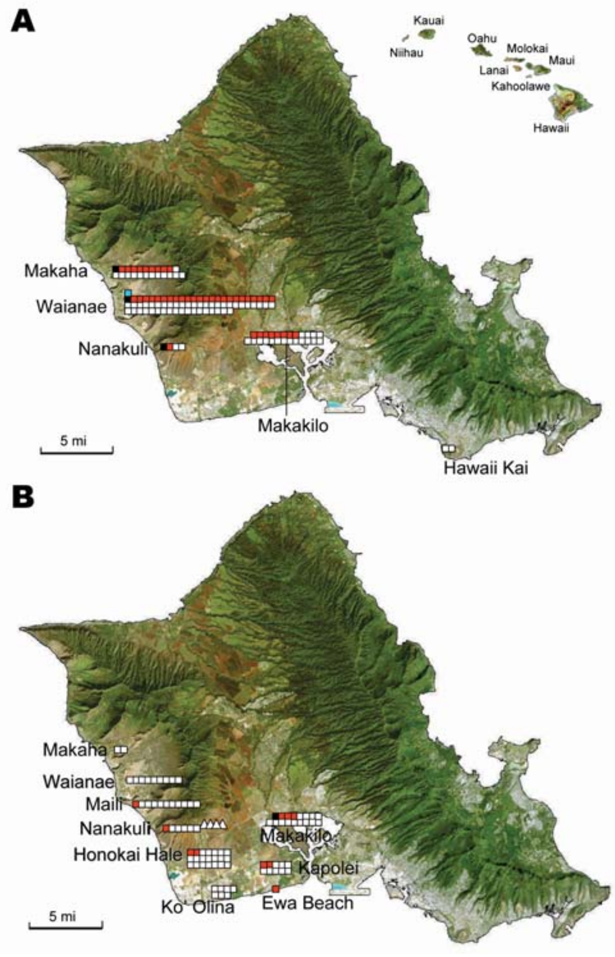

Figure. Detection of *Rickettsia typhi* and *R. felis* DNA in *Xenopsylla cheopis* trapped in Oahu, Hawaii, in A) 2004 and B) 2006 and 2007. Hawaii is shown in the inset. Symbols correspond to sites of sample collection. White squares, collections in 2004 and 2006 of fleas negative for *R. felis* and *R. typhi*; white triangles, collections in 2007 of fleas negative for *R. felis* and *R. typhi*; red squares, fleas positive for *R. felis*; black squares, fleas positive for *R. typhi*; blue squares, fleas positive for both *R. typhi* and *R. felis*. Maps were obtained from www.hear.org/starr/maps/stock/landsat.htm

Detection of *R. felis* and *R. typhi* DNA was conducted by using a TaqMan assay for the citrate synthase (*glt*A) gene of *Rickettsia* spp. (forward primer: 5′-GATTTTTTAGAAGTGGCATATTTG-3′; reverse primer: 5′-GGKATYTTAGCWATCATTCTAATAGC-3′) and species-specific probes (*R. typhi*: 5′-CalRed610-TT(T)A(C)TA(C)A(A)AG(A)T(T)G(C)T(C)A-BHQ2-3′; *R. felis*: 5′-Cy5-CTA(C)GGA(G)AATT(G)CCA-BHQ3-3′); locked nucleic acid bases, shown in parentheses, were incorporated to improve probe binding. Specificity of probes was tested by using DNA of *R. prowazekii*, *R. typhi*, and 23 spotted fever group rickettsial isolates. The Brilliant Q PCR core reagent kit (Stratagene, La Jolla, CA, USA) and an *i*Cycler (Bio-Rad, Hercules, CA, USA) were also used. Positive control plasmids contained a 265-bp target fragment from *R. typhi* strain Wilmington or *R. felis* strain LSU.

DNA from 210 *X. cheopis* fleas was examined, including 122 fleas collected from 61 mice trapped in 2004, 84 fleas from 55 mice trapped in 2006, and 4 fleas from 2 mice trapped in 2007 (Table). Victor Tin Cat Repeating Mouse Traps (Woodstream Corp., Lititz, PA, USA) were located in 10 communities on the leeward and southeast parts of Oahu, the former representing areas where typhus cases are most frequently diagnosed on this island. The largest collections were obtained from Waianae (36.6% of flea specimens), Makakilo (20%), and Makaha (11.9%); only 1 sample each was available from Ewa Beach and Hawaii Kai. An average of 1.8 (median 1) fleas was collected from each animal.

Four fleas (1.9%, n = 210) contained only *R. typhi* DNA, and 52 (24.8%) fleas contained only *R. felis* DNA. The amplicon sequences were identical to homologous sites of *R. typhi glt*A (AE017197) or *R. felis glt*A (CP000053). One flea contained *R. felis* and *R. typhi* DNA. This result was confirmed by cloning of 4 replicate amplicons and sequencing of 24 randomly selected clones. Both DNAs were confirmed to be present in each amplicon. The highest rates of fleas infected with rickettsial agents were detected in Makaha (44%, n = 23), Waianae (38%, n = 68), and Makakilo (32%, n = 25) during the 2004 collection. *R. typhi* was detected in 4 sites (Makaha, Makakilo, Nanakuli, and Waianae). All DNAs extracted from brain tissues of 55 mice collected in 2006 were PCR negative for *R. felis* and *R. typhi*.

Conclusions

We report molecular detection and identification of *R. typhi* associated with rat fleas (*X. cheopis*) collected from house mice (*M. musculus*) in western Oahu, Hawaii. *R. felis*, the etiologic agent of cat flea rickettsiosis, was also found associated with rat fleas collected from house mice.

The role of commensal rats and their fleas is often regarded as axiomatic for maintenance of murine typhus (*1*,*2*). However, other rodents and their ectoparasites have been implicated as alternative competent reservoirs and vectors of *R. typhi,* respectively (*1*,*5*). House mice are highly susceptible to experimental infection with *R. typhi*, which may establish a persistent intracerebral infection lasting for up to 5 months and is excreted in the urine (*6*). A previous study reported house mice naturally infected with *R. typhi* in the state of Georgia (*7*); however, no PCR-positive mice were detected in our study. Eruptions of mouse populations in the absence of rats have been implicated in several outbreaks of murine typhus (*1*); however, these observations were not supported by laboratory data. Early reports relied mostly on isolation of rickettsiae from tissue or fleas and serosurveys that did not necessarily provide accurate speciation of rickettsial isolates in the absence of precise molecular characteristics. Recent surveillance reports applying PCR and sequencing for detection and identification of rickettsiae have also detected *R. typhi* DNA in *X. cheopis*, *Leptosylla segnis*, and *Ctenocephalides felis* fleas in different parts of the world (*2*,*5*,*8*).

R. felis has been detected in many countries, primarily associated with *C. felis* fleas parasitizing cats, dogs, or opos-

Table. Prevalence of *Rickettsia felis* and *R. typhi* in *Xenopsylla cheopis* fleas by PCR, Oahu, Hawaii*

Location	No. (%) positive collected in 2004					No. (%) positive collected in 2006				
	Mice	Fleas	*R. felis*	*R. typhi*	*R. felis* and *R. typhi*	Mice	Fleas	*R. felis*	*R. typhi*	*R felis* and *R. typhi*
Ewa Beach	0	0	NA	NA	NA	1	1	1	0	0
Hawaii Kai	1	2	0	0	0	0	0	NA	NA	NA
Honokai Hale	0	0	NA	NA	NA	12	21	2 (10)	0	0
Kapolei	0	0	NA	NA	NA	7	10	2 (20)	0	0
Ko Olina	0	0	NA	NA	NA	3	7	0	0	0
Maili	0	0	NA	NA	NA	7	11	1 (9)	0	0
Makaha	12	23	9 (39)	1 (4.3)	0	2	2	0	0	0
Makakilo	17	25	8 (32)	0	0	13	17	3 (18)	1 (6)	0
Nanakuli	3	4	1 (25)	1 (25)	0	6	6	1(17)	0	0
Waianae	28	68	24 (35)	1(1.5)	1 (1.5)	4	9	0	0	0
Subtotal	61	122	42 (34)	3 (3)	1 (0.8)	55	84	10(12)	1 (1.2)	0
Total†	118	210						52 (25)	4 (1.9)	1 (0.05)

*NA, not available (no samples were collected).
†Includes 2 mice and 4 fleas collected in 2007 from Nanakuli; these samples were PCR negative.

sums (*2*), although *R. felis* is rarely detected in cats or opossums. Surprisingly, *R. felis* may be present in rat fleas (*X. cheopis*) as demonstrated here and in a recent report from Indonesia (*9*). The prevalence of *R. felis* ranges from 5% to 45.8% for large collections of fleas, sometimes up to 100% when small collections are tested (*8*,*10*,*11*), and is often higher when compared with the prevalence of *R. typhi*, as in our study. *R. felis* has also been detected in *Anomiopsyllus nudata* collected on white-throated woodrats (*Neotoma albigula*) (*12*). Co-infection with *R. felis* and *R. typhi* in fleas has been reported only in experimentally infected fleas (*13*). However, it is not known if either pathogen has any advantage for acquisition, life-long persistence, or transmission by fleas.

Murine typhus caused by *R. typhi* has been considered to be the only rickettsiosis present in Hawaii, but our data indicate that a second flea-borne rickettsia, *R. felis*, circulates in areas on Oahu where murine typhus is endemic. Clinical symptoms for cat flea rickettsiosis (CFR) are not agent specific and, as for other rickettsioses, include fever, headache, and rash. Antibodies against *R. felis* variably cross-react with *R. typhi*, *R. rickettsii*, and other spotted fever group rickettsia antigens (*14*). Consequently, *R. felis* infection in humans can be misdiagnosed or missed without *R. felis* antigen testing. Only a handful of cases of CFR have been reported worldwide, and only 8 cases have been specifically confirmed by PCR (*14*,*15*). The Hawaii State Department of Health reported a mean of 4.2 cases annually from 1992 through 2001 and a mean of 20 cases annually through 2006, but an outbreak of 47 cases occurred in 2002 (*3*). Because all cases of murine typhus in Hawaii were diagnosed by using potentially cross-group reactive serologic tests and not specific molecular or serologic tests, it is difficult to exclude or confirm if humans have CFR or what the relative prevalence of the 2 rickettsial diseases may be in Hawaii.

Since 25% of the fleas removed from mice were positive for only *R. felis* DNA, this pathogen may pose a serious risk to human health in Oahu. Further studies are warranted to establish the true human prevalence of murine typhus and cat flea rickettsiosis in Hawaii, to define the clinical spectrum of these infections with more specific confirmatory diagnostic tests, and to establish the role of fleas and different rodents in the epidemiology of the 2 diseases.

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New Hosts for Equine Herpesvirus 9

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Equine herpesvirus 9 was detected in a polar bear with progressive encephalitis; the source was traced to 2 members of a potential equid reservoir species, Grevy's zebras. The virus was also found in an aborted Persian onager. Thus, the natural host range is extended to 6 species in 3 mammalian orders.

Equine herpesvirus (EHV) 9, a varicellovirus in the subfamily *Alphaherpesvirinae*, is the newest member of the equine herpesviruses. EHV-9 is most closely related to the recently emergent neurotropic pathogen, EHV-1, but was first described in an outbreak of disease in Thomson's gazelles (*Gazella thomsoni*) and subsequently in a giraffe (*Giraffa camelopardalis reticulata*) with encephalitis (*1–3*; M. Kiupel, pers. comm.). Initial findings of the virus' virulence and potential for transmission between equids and artiodactyls were alarming and provided the impetus for experimental studies, which showed that disease could be induced in members of an additional 8 mammalian taxa: dogs, cats, horses, mice, hamsters, pigs, goats, and marmosets (*4–6*).

Preliminary data suggest that equids are natural hosts of EHV-9 and experience little or no illness when infected. Seroconversion was detected in 60% of wild Burchell's zebras (*Equus burchelli*) in Tanzania without any associated illness (*3*). When domestic horses (*E. caballus*) were infected by intranasal inoculation with 10^7 PFU of EHV-9, only mild disease developed and clinical signs were limited to transient fever (*6*). Natural coadaptation of EHV-9 and equids was corroborated by the severity of disease seen in nonequids: fulminant encephalitis with extensive neuronal necrosis in both spontaneous cases and experimental models (*2–6*). Antigenic and genetic similarities of EHV-9 with other equine herpesviruses are, likewise, consistent with the theory that equids are primary hosts. However, active infection has never been conclusively documented in any member of the Equidae (*2–6*).

The potential vulnerability of diverse species to EHV-9 has raised concern about the virus as an anthropozoonotic pathogen (*2–6*). Of additional note is the lack of known reservoir species and infectivity in nonlaboratory environments. We report natural EHV-9 infection and resultant disease in an ursid and 2 equid species, confirming the virus' promiscuity and pathogenicity and supporting its natural residence in wild equids.

The Study

In July, 2007, a 12-year-old polar bear (*Ursus maritimus*) in a zoological garden in San Diego, California, showed progressive neurologic signs that were refractory to therapy. The animal was housed ≈200 feet from a herd of recently relocated Grevy's zebras (*E. grevysi*). Ultimately, the bear was euthanized. Postmortem examination showed nonsuppurative meningoencephalitis with neuronal and glial intranuclear inclusion bodies (Figure 1, panel A). PCR targeting of conserved regions of herpesvirus DNA polymerase genes identified a virus in the brain with 98% homology to EHV-1 in a 165-bp segment of DNA (*2*,*7*). Subsequently, 2 other PCRs targeting an additional 742 bp of the DNA polymerase gene (sense primer 5′-GCATYWTCCCCCCGTTKATRAC-3′ and antisense primer 5′-ATAGYSAARRCCACGCCTTY-3′) and 1,181 bp of the glycoprotein B (gB) gene (sense primer 5′-CTTGTGAGATCTAACCGCAC-3′ and antisense primer 5′-GGGTATAGAGCTTTCATGGGG-3′) identified the virus as EHV-9 and enabled more precise strain determination (*2*). DNA segments of the terminase and gB genes were also characterized to generate additional phylograms and to compare with molecular findings from other animals in this study (*8*,*9*).

Before the polar bear case, EHV-9 had been detected at the same zoological garden in 2 Grevy's zebras from the same herd, which had been relocated near the polar bears. One of the infected Grevy's zebras was 8 days old and had viral interstitial pneumonia; the other was an adult with rhinitis and intranuclear inclusion bodies (Figure 1, panel B). Both zebras were immunocompromised as a result of other concurrent conditions (i.e., sepsis, diarrhea, and tracheitis in the neonate and a traumatic nonhealing wound with fungal infection in the adult). EHV-9 was also found by a retrospective analysis of tissues from an aborted Persian onager (*E. hemionus onager*) fetus from a zoological park in Washington, DC (*10*). The onager fetus was aborted after the dam came in close proximity to a Grevy's zebra (*10*). PCR and DNA sequencing analyses of the DNA polymerase showed that the zebras and the onager had an EHV-9 strain identical to that found in the polar bear. PCR results for other potential pathogens (e.g., EHV-1, adeno-

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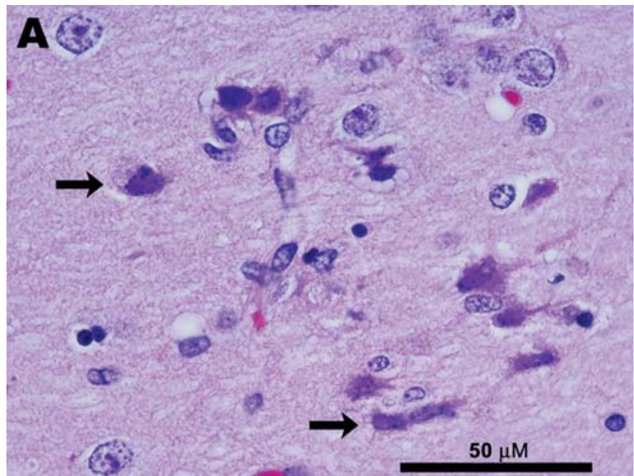
virus, chlamydiae, rickettsiae, rabies, paramyxovirus, and West Nile virus) were negative in the polar bear, zebras, and onager.

To test the possibility that other reservoirs of EHV-9 exist, we conducted a molecular survey using herpesvirus consensus-based PCRs for DNA polymerase, terminase, and gB gene segments and EHV-9–specific PCRs. Samples were blood and nasal swabs from a Damara's zebra (*E. burchellii antiquorum*), Somali wild ass (*E. asinus somalicus*), and eastern kiang (*E. kiang holdereri*) from the aforementioned San Diego zoological park (*6–8*). Novel herpesviruses, but not EHV-9, were detected in all 3 equid species. Distance analyses of DNA and predicted amino acid sequences from these novel herpesviruses, EHV-9, and previously described viruses from a variety of animals were done by using PAUP (*11*). Phylograms from neighbor-joining distance and parsimony methods showed a partitioning of the equid herpesviruses into 2 clades, representing alpha- and gammaherpesviruses. One clade included EHV-9, EHV-1, and EHV-4 and was paraphyletic to herpesviruses from primates; the other clade comprised equid herpesviruses and a hyena herpesvirus that branched separately from other viruses (Figure 2). Phylograms derived from gB and terminase gene segments, individually or concatenated, produced comparable results. GenBank accession numbers for all sequences from this study are EU17146–EU17156.



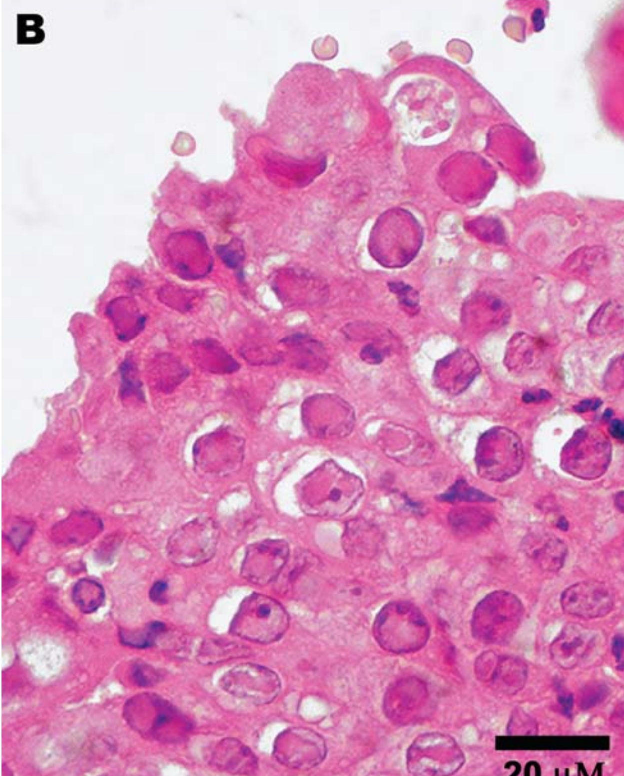


Figure 1. Photomicrographs showing A) encephalitis with neuronal necrosis and intranuclear inclusions (arrows) in a polar bear (*Ursus maritimus*); scale bar = 50 µm; hematoxylin and eosin stain; and B) Grevy's zebra (*Equus grevysi*) with acute rhinitis with eosinophilic inclusions in respiratory epithelium; scale bar = 20 µm; hematoxylin and eosin stain.

Conclusions

This report demonstrates interspecies transmissibility of EHV-9 on an ordinal level and confirms the virus' neurotropic pathogenicity in nonequids. Our data show that EHV-9 is able to naturally infect and cause encephalitis in polar bears that had no direct contact with an animal point source. Previous reports described EHV-9 infection and encephalitis in Thomson's gazelles and a reticulated giraffe that directly commingled with zebras (*2*, *12*; M. Kiupel., pers. comm.). By contrast, the polar bear from our study was probably infected by a fomite contaminated by an adjacent herd of Grevy's zebras. Animal-to-animal transmission, although possible, is extremely unlikely because of the space separating the polar bear and zebra enclosures. In either instance (direct animal-to-animal or indirect fomite transmission), the infectivity of EHV-9 for polar bears would have been substantial.

Our data also demonstrate naturally occurring EHV-9 infection in equids and suggest that ≥1 species of zebra may be hosts. Findings point toward Grevy's zebras as 1 reservoir species. In addition to finding active infection with EHV-9 in the zebras, the nature of the lesions and association of disease with compromised immunity and a perinatal animal emulated the host–pathogen dynamics of EHV-1 in domestic horses (*13*). A prior serologic survey that used virus-specific neutralization assays suggested that Burchell's zebras may also be natural hosts of EHV-9, although features of active infection, such as latency and viral shedding, were not investigated in that study (*3*). Excluding experimental studies, neither titers to EHV-9 nor the virus itself have been found in domestic horses (*6*).

An additional finding was the taxonomic affinity of

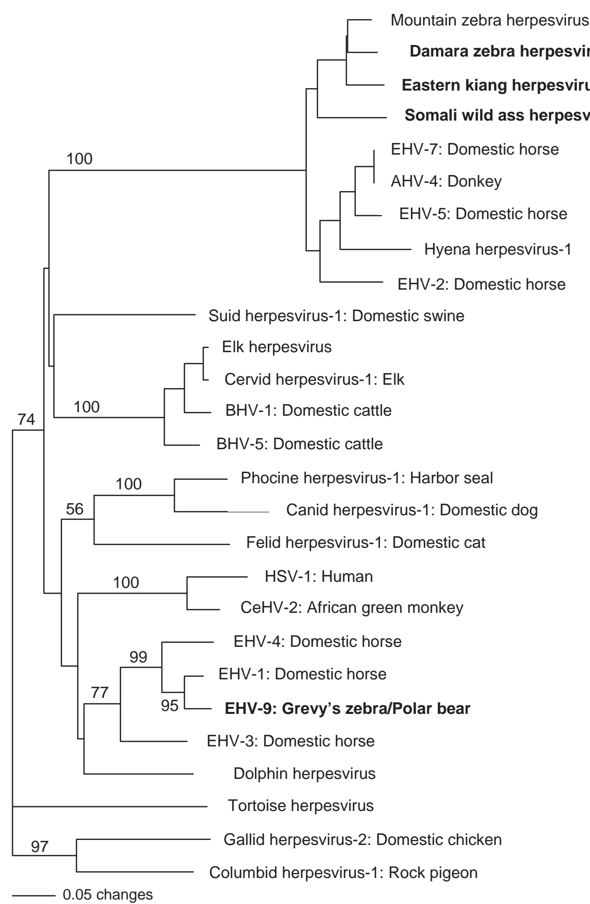


Figure 2. Phylogram of all equine herpesviruses and related viruses from other animals and their respective hosts created from a predicted amino acid segment of the DNA polymerase gene. All sequences obtained in this study are in **boldface**; bootstrap values >1,000 replicates are denoted. Note clustering of equine gammaherpesviruses and paraphyletic grouping of equine herpesviruses (EHV) -1, EHV-4, and EHV-9 with primate herpesviruses and dolphin herpesvirus. Sequence accessions, sources, and abbreviations are available online (www.cdc.gov/EID/content/14/10/1616-F2.htm)

EHV-9 and other equine alphaherpesviruses to herpes simplex virus 1 and cercopithecine herpesvirus 2. EHV-1 is endemic in domestic horses and can cause pneumonia, myeloencephalitis, and abortion (*13*). EHV-9 is highly related to EHV-1 but unique in its ability to cause disease in a variety of other mammals, including primates (*1–5*). This ability has drawn attention to the possibility of eventual zoonotic transmission and supports grading of EHV-9 with other incipient semigeneralist pathogens (*4–6*).

Redefining the host range of EHV-9 raises new issues regarding the anthropogenic effects of assembling diverse species in zoological gardens and the growing interface between wildlife, domesticated animals, and humans. Through comparative studies with other equine herpesviruses such as EHV-1, EHV-9 can be now considered of preeminent value as a model for understanding how viruses cross species barriers.

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Extended Sequence Typing of *Campylobacter* spp., United Kingdom

Kate E. Dingle, Noel D. McCarthy, Alison J. Cody, Tim E.A. Peto, and Martin C. J. Maiden

Supplementing *Campylobacter* spp. multilocus sequence typing with nucleotide sequence typing of 3 antigen genes increased the discriminatory index achieved from 0.975 to 0.992 among 620 clinical isolates from Oxfordshire, United Kingdom. This enhanced typing scheme enabled identification of clusters and retained data required for long-range epidemiologic comparisons of isolates.

Human campylobacteriosis remains a global public health problem. Although many risk factors for this foodborne zoonotic disease are known, the relative contributions of different transmission routes are poorly quantified. Furthermore, the sources of particular infections are frequently obscure and outbreaks are rarely detected (*1*). The high genetic and antigenic diversity of the 2 major causes of campylobacteriosis, *Campylobacter jejuni* and *C. coli*, have proved to be obstacles in routine surveillance, outbreak identification, and source attribution.

Nucleotide sequence–based isolate characterization methods such as multilocus sequence typing (MLST) successfully catalog bacterial pathogens and provide a rational, definitive, and portable typing method with complete reproducibility among laboratories (*2*). Because many of the sequence types (STs) or their close relatives are observed on multiple occasions with wide geographic distribution, MLST is highly effective for long-range epidemiologic studies (*3*). However, this characteristic can limit the application of MLST to outbreak identification (*4*). We combined MLST data with sequence data of the short variable region (SVR) of the *flaA* and *flaB* loci, previously used to type *Campylobacter* spp./isolates (*5*,*6*), and a novel typing system based on *porA* locus encoding the variable outer membrane protein PorA. The resultant high-resolution 10-locus typing scheme was used to characterize 620 isolates obtained from 584 persons with human campylobacteriosis from September 2003 through September 2004 in Oxfordshire (population ≈600,000), United Kingdom; 36 isolates obtained by sampling the same patient more than once were used to confirm reproducibility.

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The Study

A comparison of our results with published population-based 7-locus MLST studies showed that the relative abundance of different clonal complexes in northwestern England from April 2003 through March 2004 was similar (*7*), presumably reflecting widely distributed foods in the United Kingdom. An exception was ST-574 complex, the central genotype, which represented >5% of cases in Oxfordshire but was absent from northwestern England. The clonal complex distribution of 171 isolates collected in New South Wales, Australia (*8*), was distinct from the 2 English datasets, although many clonal complexes were present in all 3 datasets. Fewer clonal complexes, with different relative abundances, were present in a dataset from Curaçao (*9*), likely because of different infection sources in the Dutch West Indies (Figure 1).

The differences in relative abundance of clonal complexes were mirrored by F_{ST} values calculated from concatenated nucleotide sequences of the MLST loci, which indicated that the Australian dataset was 3.6% differentiated and the Curaçao set 9.9% differentiated from the Oxfordshire dataset. One clonal complex detected in Curaçao was absent in the United Kingdom (ST-41 complex) and 13 of the clonal complexes detected in the United Kingdom were absent in Curaçao.

The antigen loci added further resolution to the Oxfordshire dataset: 575 (98.6% of isolates) *flaA* SVR sequences contained 130 distinct SVR alleles, and 567 (97.1%) *flaB* sequences contained 111 SVR alleles. The allele fragment ≈630bp used for *porA* typing was amplified and sequenced with primers MOMP-1 (5′-GAT GGT TTA ACT CTA

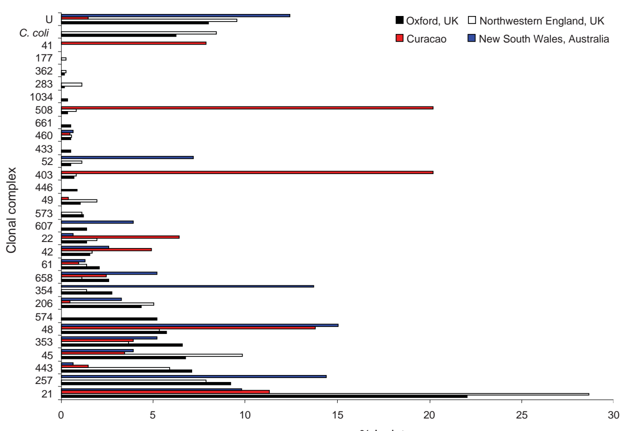

Figure 1. Relative abundance of clonal complexes of *Campylobacter* spp. detected in Oxfordshire, United Kingdom, during a 1-year study compared with clonal complexes detected in 3 other studies of human *Campylobacter* spp. infections in northwestern England (*7*), New South Wales, Australia (*8*), and Curaçao (*9*).

GCT GC-3′) or MOMP-3 (5′-GAT GGT TTA GTW GGM ACA GG-3′) and MOMP-2 (5′-TGA GAA GTT AAG TTT TGG AG AG-3′). *PorA* allele and variant numbers were assigned and sequences were deposited in a database (http://hercules.medawar.ox.ac.uk/momp). A description of amplification conditions is also available at this website. Of the 558 (95.5% of the isolates) *porA* sequences assigned, 1 occurred in 65 isolates whereas 135 alleles occurred only once.

The discriminatory index (DI) (*10*) was calculated for various subsets of these data. Each additional antigen gene increased discrimination relative to MLST data alone. The combination of *flaA* SVR and *flaB* SVR (DI = 0.976) provide a similar level of discrimination as *porA* (DI = 0.972). The DI obtained with the *porA* gene fragment alone was similar to that obtained previously with a larger fragment of the same gene (DI = 0.973) (*11*). The 10-locus combination provided a degree of discrimination (DI = 0.992) higher than those published for pulsed-field gel electrophoresis fingerprinting, antigen typing, or MLST (*4*,*6*,*9*,*12*,*13*). These studies calculated DI, ignoring the fact that some isolates probably shared a common source and may have underestimated the true DI, the capacity to discriminate between epidemiologically unrelated isolates. For the same reason, our study is also likely to have underestimated the true DI.

There were 68 groups of ≥2 isolates with identical 10-locus types, ranging in size from 2 to 34 isolates and accounting for 283 (48.5%) of the independent isolates. Of the remaining isolates, 290 (49.7%) had unique types, typing data were incomplete for the remaining 10 isolates (1.7%). A permutation test with 283 isolates belonging to a cluster showed highly significant temporal clustering of *Campylobacter* spp. isolates of identical genotype (p<0.0001). The extent of clustering was independent of group size (data not shown). Of 16 groups of ≥5 identical isolates, 5 exhibited significant temporal clustering (Table, Figure 2). Isolates belonging to the largest of these groups, comprising 34 isolates of ST-257, *flaA* 16, *flaB* 301, and *porA* 1, were submitted mainly in the last part of the study year with a peak of 5 isolates in week 39 (Figure 2). The second largest group, comprising 13 isolates, shared ST-51 and an identical *porA* type with a genotypic group comprising 7 isolates, but the 2 groups were distinct at both *flaA* and *flaB* loci. All members of these 2 groups were isolated from week 14 through week 40. Of the 12 isolates comprising the third largest group, all but 1 were isolated over a 17-week period (week 2 through week 18) at the beginning of the year (Figure 2). The smallest group of isolates to show evidence for temporal clustering comprised 5 identical isolates obtained during weeks 18–29. Some of the other genotypic groups were seen throughout the year, with no evidence for temporal clustering; for others, weak evidence of clustering was found (Table).

Table. Temporal association of genotypically identical isolates of *Campylobacter* spp., United Kingdom*

ST	10-locus genotype			No. isolates	p value of temporal association
	flaA SVR	*flab* SVR	*porA*		
49	11	11	53	5	0.0005
206	14	96	7	5	0.36
583	239	177	43	5	0.85
45	8	8	44	6	0.92
48	32	103	14	6	0.44
354	18	18	57	6	0.42
51	21	21	10	7	0.0007
475	105	105	67	7	0.16
50	36	36	6	8	0.35
827	255	236	33	8	0.49
19	36	36	7	9	0.15
658	5	5	25	9	0.19
104	36	36	14	11	0.14
574	105	105	1	12	<0.0001
51	316	295	10	13	<0.0001
257	16	301	1	34	<0.0001

*ST, sequence type; SVR, short variable region.

Conclusions

Clusters were detected by this method but represented a small part of the overall disease incidence (*14*). Temporal association within these clusters suggests that they may represent undetected outbreaks. Further epidemiologic information unavailable to this study, which was based solely on laboratory isolates, would be needed to confirm or refute this possibility.

The clusters occurred over periods longer than the typical duration of outbreaks of gastroenteritis, which was consistent with episodes of contamination entering the food chain rather than single proximate-point source events.

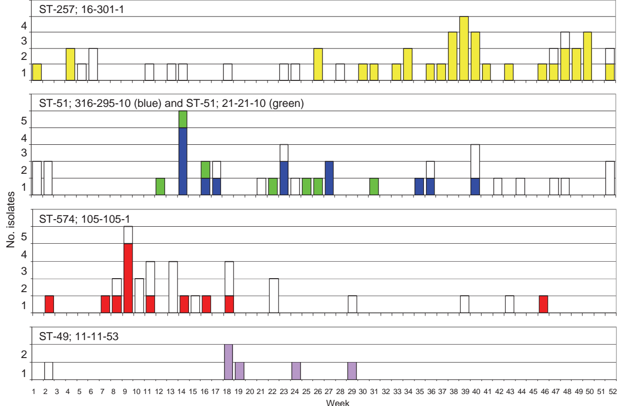

Figure 2. Clusters of related 10-locus types of *Campylobacter* spp. detected in Oxfordshire, United Kingdom, during a 1-year study. Five groups of isolates with identical genotypes show statistically significant clustering in time (p values are shown in the Table). Each group is indicated by 1 color. White bars indicate other isolates that share the same sequence type (ST) but that are differentiated by their different antigen type. Numbers of isolates of each genotype are shown on a weekly basis; week 1 corresponds to the start of the study on September 15, 2003.

These findings indicated that many of these clusters may be associated with widely distributed foods. The observed temporal association of groups of identical isolates could also be caused by certain genotypes having different seasonality or environmental sources from sporadic cases. In either scenario, the data supported the interpretation that cases sharing an identical genotype were more likely to be epidemiologically linked than those infected with different genotypes.

In conclusion, the 10-locus typing scheme is highly discriminatory for *C. jejuni* and *C. coli* isolates and provides information that can be used flexibly to support long-range comparisons and short-term epidemiology. The scheme can be applied in real time or near real time, enabling the data to be used to identify outbreaks and inform public health interventions. When combined with improved genetic methods of attributing source of bacterial isolates (*15*), this approach will contribute to refining the epidemiology of these enigmatic pathogens.

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Chikungunya Outbreak, South India, 2006

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We investigated chikungunya outbreaks in South India and observed a high attack rate, particularly among adults and women. Transmission was facilitated by *Aedes aegypti* mosquitoes in peridomestic water containers, as indicated by a high Breteau index. We recommended vector control measures and health education to promote safe water storage practices.

Chikungunya fever (CHIK) outbreak was observed in India in December 2005. Phylogenetic analysis of the isolated virus showed a central-east African strain that was closely related to the strain from the Reunion Islands (*1*). Historically, the first outbreak of CHIK was reported in 1963 in Kolkata (*2*), and the last reported outbreak occurred in 1973 in Maharashtra (*3*). The reemergence of the virus may have been facilitated by human population migrations in the Indian Ocean region (*4*). Since December 2005, cases of CHIK were reported from several Indian states including Andhra Pradesh, Maharashtra, Karnataka, Tamil Nadu, and Madhya Pradesh. We investigated CHIK outbreaks in Andhra Pradesh and Tamil Nadu to describe the outbreak, estimate the incidence of subclinical infection, and propose control measures.

The Study

In Andhra Pradesh, on the basis of the reports obtained from the local primary health centers, we selected Mallela village (2006 population: 1,965) of Kadapa district. In Tamil Nadu, we investigated the outbreak in Gowripet area (2006 population: 2,649) of Avadi, a suburban locality of Chennai City where a large number of persons with fever and joint pain were reported in June 2006.

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In both settings, we conducted a door-to-door search of all households for case-patients who had acute onset of febrile illness and joint pain. We described the outbreak in terms of time, place, and person. We collected blood samples from case-patients after obtaining informed consent. We also collected blood samples from consenting asymptomatic persons in Mallela village to assess the incidence of subclinical infections. We tested serum samples for immunoglobulin (Ig) M antibodies against CHIK virus using IgM-capture ELISA at the National Institute of Virology, Pune (*1*).

For the larval survey in Mallela, we selected a representative sample of households after stratifying the village by attack rates; in Avadi, we surveyed all households. We calculated house index (HI), the proportion of houses having containers with larvae, and the Breteau index (BI), the number of containers positive for mosquito larvae per 100 houses. In Avadi, we mapped all the case households using a geographic positioning system device (ArcGIS version 8.02; ESRI, Redlands, CA, USA). We divided the area into 200-m^2 grids to determine correlation between BI and attack rates.

We identified 242 case-patients meeting the case definition in Mallela (attack rate: 12%; no deaths) and 575 case-patients in Avadi (attack rate 22%; no deaths). The median number of joints affected in Mallela and Avadi was 3 and 4, respectively. The most common joints involved were ankle, knee, wrist, and small joints of hands in both settings. In addition to fever and joint pain, 59 (10%) and 28 (12%) case-patients in Mallela and Avadi, respectively, reported a rash. Case-patients were bed-ridden for an average of 6 to 7 days in both settings.

Attack rates were higher among persons ≥15 years of age and females in both settings (Table). In Mallela, cases began occurring during December 2005 and peaked during the first week of March 2006 (Figure 1). In Avadi, cases began occurring during May 2005 and peaked during the third week of June 2006 before declining. In Mallela, attack rates in different areas of the village ranged between 0% and 21%. Neighborhoods where persons of lower socioeconomic status resided in households with a single room and no water storage facility had the lowest attack rates. In contrast, neighborhoods where people lived in pucca houses (houses made with brick and mortar) with plenty of water storage containers had higher attack rates. Both outbreaks occurred during the summer months with temperatures ranging from 30°C to 44°C in Mallela and 27°C and 37°C in Avadi.

We conducted a larval survey in 56 houses in Mallela and all 657 households in Avadi. In both settings, water was scarce and residents used a variety of water storage containers, including plastic/earthen pots, plastic drums, and cement cisterns. The mean number of containers per household was 9

Table. Characteristics of chikungunya outbreak in South India, 2005–2006*

Area	Mallela, Andhra Pradesh	Gowripet, Avadi, Tamil Nadu
Setting	Rural	Urban
Population	1,965	2,649
Attack rate		
Overall	12% (n = 242)	23% (n = 575)
Age, y		5.8%
0–4	2.4%	20%
5–14	3.6%	24%
15–44	12.9%	22.8%
≥45	22.2%	
Gender		18.9%
M	9.9%	
F	14.8%	24.5%
Chikungunya-specific IgM positivity	67% (90/134)	56% (5/9)
Subclinical infection		
No. tested	100 in all age groups	ND
Age-specific prevalence of IgM antibodies		
5–14 y	4%	ND
15–44 y	8%	ND
≥45 y	3%	ND

*Ig, immunoglobulin; ND, not done.

in Avadi. The HI and BI were 30% (17/56) and 39% (22/56), respectively, in Mallela. The HI and BI were 23% (148/657) and 35% (228/ 657), respectively, in Avadi. We observed a weak but significant correlation between attack rates by 200-m^2 grids and BI ($r = 0.37$, $p = 0.04$) (Figure 2).

In Avadi, 5 of the 9 blood samples collected from the case-patients were positive for IgM antibodies. In Mallela, 90 (67%) of the 134 blood samples collected from case-patients were positive for IgM antibodies. In Mallela, we included 100 asymptomatic volunteers for serologic testing (median age 25 years, range 6–70); 33 were <15 years of age and 51 were female. Fifteen of the 100 asymptomatic persons had detectable IgM antibodies against CHIK virus. Our findings suggest that the apparent to inapparent case ratio was almost 1:1. There was no significant difference in the prevalence of IgM antibodies between age groups ≥15 years of age and <15 years of age (16% vs. 12%; $p = 0.40$) or between sexes (16% in males vs. 14% in females, $p = 0.47$).

Conclusions

The key finding of our Chikungunya outbreak investigation was a high attack rate, particularly among adults and females. The outbreak occurred nearly 32 years after the last reported outbreak of CHIK in 1973 and was characterized by a prolonged duration. Transmission was facilitated by larvae of *Aedes aegypti* mosquitoes in peridomestic water containers. Our findings also suggest a considerable number of subclinical infections during the outbreak.

The explosive nature of the outbreak with high attack rates might be due to the absence of herd immunity to the central/East African genotype of the CHIK virus isolated in India and other countries in Indian Ocean region. CHIK outbreaks reported in the 1960s and 1970s from India were related to the Asian genotype of the virus (*1*). Unlike with dengue fever, we observed higher attack rates among adults than among children. We observed higher attack rates in females as observed in other countries (*5–7*).

The epidemic curves observed in both study sites indicated that transmission was ongoing for a considerably long period. The short flight range of the vector likely resulted in gradual transmission of infection among hosts in both communities.

The BI threshold for predicting CHIK transmission in India is not available. However, in both areas, BI was higher than 5 (35%–39%), which is the threshold for dengue transmission per the guidelines of the National Institute of Communicable diseases, India (*8*). The weak positive correlation between attack rate and BI in Avadi could be due to high vector density, which might distort the association between vector indices and clinical CHIK.

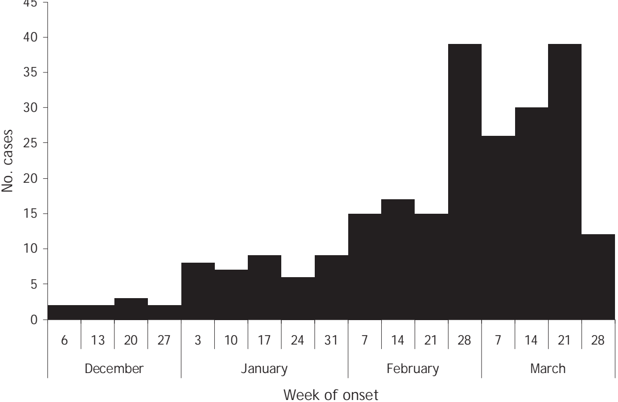

Figure 1. Chikungunya cases by week of onset, Mallela village, Kadapa district, Andhra Pradesh, India, 2005–2006.

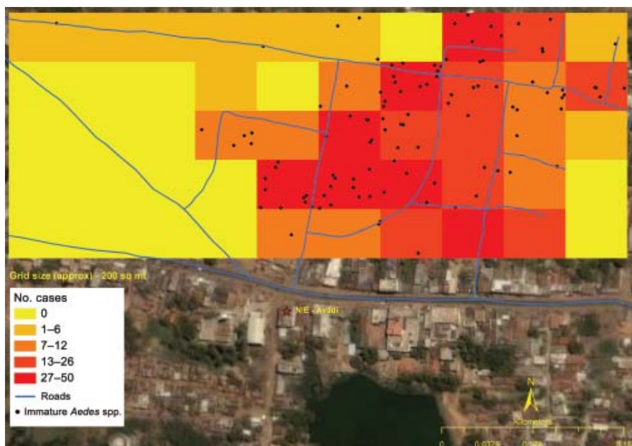

Figure 2. Chikungunya cases and presence of *Aedes* spp. immature mosquitoes, Gowripet, Avadi, Chennai, Tamil Nadu, India, 2006.

The limitations of our study were the lack of uniform methods for investigating 2 outbreaks and the convenient sampling method used to estimate incidence of subclinical infection. CHIK outbreaks occurred during the peak summer season because of favorable environment for *Ae. aegypti* mosquitoes in the form of water storage containers that were used in the absence of regular water supply and acute water scarcity. Most of these containers were either uncovered or partially covered and were not cleaned at regular intervals. Thus, a regular water supply that negates the need for water storage, education of the public for safe water storage measures, and environmental control are much needed public health measures to combat future CHIK outbreaks.

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Dr Kaur completed her postgraduate training in general medicine in 2004. She joined the National Institute of Epidemiology as a research officer in 2004 and is currently a second-year student in the Field Epidemiology Training Program at the National Institute of Epidemiology, Chennai, India. Her research interests are epidemiology of noncommunicable diseases, health services research, and applications of spatial methods for epidemiologic studies.

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Control Strategy for *Echinococcus multilocularis*

Daniel Hegglin and Peter Deplazes

Echinococcus multilocularis, the causative agent of zoonotic alveolar echinococcosis, can be controlled effectively by the experimental delivery of anthelminthic baits for urban foxes. Monthly baiting over a 45-month period was effective for long-lasting control. Trimonthly baiting intervals were far less effective and did not prevent parasite recovery.

Human alveolar echinococcosis (AE) is a potentially fatal zoonosis (*1*) caused by *Echinococcus multilocularis*, a helminth that is widespread in red foxes (*Vulpes vulpes*). In recent years, fox populations in central Europe have increased and extended their habitats into urban areas (*2*). Consequently, AE rates have increased (*1*). Epidemiologic and ecologic studies have demonstrated that urban peripheries provide suitable conditions for high densities of susceptible final hosts (red foxes) and intermediate hosts (voles) (*3*). Consequently, these densely populated areas can be highly contaminated with *E. multilocularis* eggs and are of special interest for the development of cost-effective control strategies (*2*).

Parasite control by substantially reducing intermediate host density (rodents) or by large-scale fox culling are far less promising strategies than delivering anthelminthic baits for foxes (*4*). In 3 studies from southern Germany, the regular distribution of 15 to 50 praziquantel baits per km^2 resulted in a significant decrease in the prevalence of foxes from 35%, 64%, and 14–37% to 1%, <20%, and 2%–12%, respectively (*5–7*). Different baiting studies in Japan have demonstrated lower environmental contamination with *E. multilocularis* eggs when praziquantel baits are placed around fox dens (*8*), along roads, and in forests that provide protection from the wind (*9*). Even in small-scaled areas of ≤6 km^2, anthelmintic baiting has been effective (*10,11*), resulting in a lower reinfection rate in foxes because of a lower prevalence of the parasite in intermediate hosts (*11*). However, local eradication of the parasite is difficult to achieve. Long-lasting interventions seem to be necessary for effective control of *E. multilocularis* (*12*). Therefore, cost-effective baiting strategies must be designed.

Our aim was to provide data so that optimal and cost-effective baiting strategies could be designed. To obtain these data, we investigated the effect of different baiting intervals and the postcontrol recovery of the parasite population.

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The Study

The study, conducted during 1999 and 2007, was designed as a follow-up to an experimental field study that had been conducted in the conurbation of Zurich (*11*). Along the urban periphery, we selected 12 study plots of 1-km^2 each and 1 additional, 6-km^2, plot. Experimental bait delivery was structured in 2 phases: April 2000–October 2001 and November 2001–December 2003. Within these 2 periods, a total of 5 different treatment schemes were used in the study plots: 1) no bait delivery during the whole study (co/co); 2) monthly bait delivery during the first phase and trimonthly delivery during the second phase (b1/b3); 3) no bait delivery during the first phase and trimonthly delivery during the second phase (co/b3); 4) monthly bait delivery during the first phase and no delivery during the second phase (b1/co); and 5) monthly bait delivery during the first phase and the second phase (b1/b1) (Figure 1).

We used commercial fox baits (Impfstoffwerk Dessau Tornau GmbH, Rosslau, Germany) that contained 50 mg of the anthelminthic praziquantel (Droncit Bayer AG, Leverkusen, Germany). At each baiting interval, 50 baits per km^2 were distributed manually at places that were most likely to be frequented by foxes (*11*).

Using a sandwich ELISA, we determined the effect of baiting by detecting *E. multilocularis* coproantigens in fox feces (*13*). Feces samples were collected at least once per month in all study plots during sampling period 1 before and during the initial phase of bait delivery (November 1999–June 2000), sampling period 2 (July–October 2001), and sampling period 3 (September–December 2003). Additionally, during November 2006–January 2007 (sampling period 4), feces samples were collected in the three 1-km^2 plots that had never been baited and in the treated section of the 6-km^2 plot that had been baited monthly throughout the entire baiting period (Figure 1).

The proportion of coproantigen-positive feces detected by ELISA (hereafter referred to as *E. multilocularis* contamination) showed no significant statistical variation in the three 1-km^2 plots that were never baited and averaged 26.5% contamination (Figure 2). In the 6 areas that were baited at monthly intervals during the first phase, *E. multilocularis* contamination decreased significantly from sampling period 1 (b1/b3 plots: 22.2%, 95% confidence interval [CI] 11.2%–37.1%; b1/co plots: 37.7%, CI 26.3%–50.2%) to sampling period 2 (b1/b3 plots: 5.4%, CI 2.4%–10.4%; b1/co plots: 5.6%, CI 2.3%–11.2%) (Figure 2). After reassigning 3 monthly baited plots to control plots (b1/co plots), a significant rise of *E.multilocularis* contamination was detected to 30.2% (CI 24.9%–36.0%). Also,

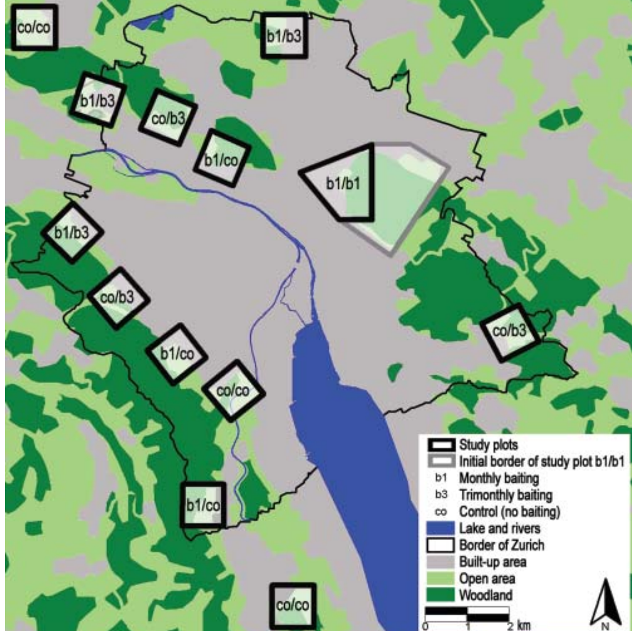

Figure 1. Study area of the anthelminthic baiting experiments in the conurbation of Zurich, Switzerland. Thirteen study plots were defined along the urban periphery during the 2-phased baiting period (phase 1, April 2000–October 2001; phase 2, November 2001–December 2003). Five different treatment schemes were used in these plots: co/co = no bait delivery during the whole study (n = 3 sites of 1 km^2); b1/b3 = bait delivered monthly during the first phase and trimonthly during the second phase (n = 3); co/b3, no bait delivery during the first and trimonthly delivery during the second phase (n = 3); b1/co, monthly bait delivery during the first and no delivery during the second phase (n = 3); b1/b1, monthly bait delivery during the first and the second phase in a single study plot. This largest study plot comprised initially an area of 6 km^2 (gray line) and finally an area of 2 km^2 during the second baiting phase.

the change from a monthly baiting interval to a trimonthly interval (b1/b3 plots) resulted in a significant increase to 19.5% (CI 15.2%–24.4%), whereas the trimonthly bait delivery in the co/b3 plots led to a significant decrease from 30.4% (CI 22.9%–38.8%) to 17.6% (CI 13.3%–22.5%).

Three years after the end of bait delivery, *E. multilocularis* contamination in the 3 co/co plots remained on a similar level (15.4%, CI 8.7%–24.5%). In contrast, *E. multilocularis* contamination was still low (3.1%, CI 0.6%–8.7%) in the b1/b1 plot. The 3 coproantigen-positive feces samples did not contain taeniid eggs.

Conclusions

This 6-year experimental field study shows basic information about the susceptibility of *E. multilocularis* to small-scale control strategies targeted to high disease-endemic foci in urban areas. Monthly, local delivery of anthelmintic baits reduced *E. multilocularis* contamination to a low level. Our experimental model shows that although the trimonthly baiting intervals reduce such contamination significantly, the reduction is far less than that achieved by baiting at monthly intervals. In addition, trimonthly baiting did not help to maintain the low *E. multilocularis* contamination achieved by the previous 1.5 years of intensive monthly baiting.

Our finding contrasts with the results of 2 German studies: in rural, large-scale study areas where baits were distributed once every 3 months during 9 and 18 months, respectively, baits were effective in maintaining the prevalence of *E. multilocularis* in foxes at the same level as that in previous baiting campaigns conducted at intervals of 6 weeks (*6,7*). It is possible that lower fox densities in rural areas compared with urban areas or the shorter periods during which trimonthly baiting intervals were applied are responsible for this difference. More likely, the difference can be explained by border effects. Foxes with home ranges near the border of a baited area have less access to the anthelminthic baits. Furthermore, immigrating foxes can contribute to *E. multilocularis* contamination. In large baiting areas a smaller proportion of treated foxes live near the border of the treated area and a smaller proportion of foxes are immigrated foxes. Therefore, small baiting areas are more affected by border effects. Further decreasing the baiting frequency to 6-month intervals and discontinuing bait distribution caused a surge of *E. multilocularis* prevalence in foxes to the precontrol level within 36 months (*6*).

E. multilocularis contamination in nonbaited areas (co/co plots) was higher during period 3 than during period 4 (Figure 2). Natural fluctuations possibly contributed to

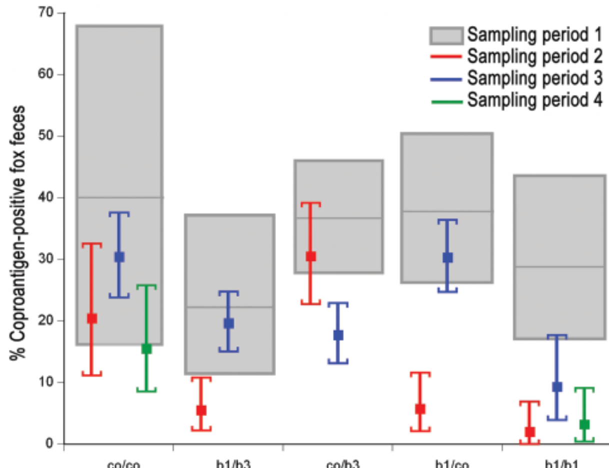

Figure 2. Contamination with *Echinococcus multilocularis* shown in study plots. Portion of coproantigen-positive (by ELISA) fox feces in study plots with 5 different treatment schemes (see Figure 1). Gray outlined boxes and error bars represent the 95% confidence intervals of ELISA-positive feces during the 4 sampling periods. Treatment schemes: co/co, control/control; b1/b3, monthly/trimonthly baiting; co/b3, control/trimonthly baiting; b1/co, monthly baiting/control; and b1/b1,monthly baiting/monthly baiting.

generally low *E. multilocularis* prevalences during the latter period. However, it was surprising that contamination was still very low 3 years after all bait delivery was ended in an area that was baited monthly during a 3.5-year period. Before baiting started, *E. multilocularis* was highly prevalent in this plot, and prevalences in European water voles (*Arvicola terrestris*) ranged from 9% to 21% during several years (1997–2000) (*11,13*). We therefore assume that a long-lasting control effect was achieved by local removal of the parasite by monthly anthelmintic bait delivery over several years.

Our results confirm the high potential of *E. multilocularis* to recover to a precontrol level within a short period if the parasite was not completely removed, as has been predicted by modeling studies (*14,15*) and shown in a single, but large, baiting area in Germany (*6*). On the basis of a spatially explicit model, Hansen et al. recommend continued bait delivery with intervals from 4 to 6 weeks (*15*). Our results support these findings. We therefore suggest continued intense baiting even if parasite contamination has been substantially reduced. Furthermore, our results provide evidence that, once the parasite has disappeared locally, recolonization can take several years, even in a small-scale baiting area.

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Mapping the Probability of Schistosomiasis and Associated Uncertainty, West Africa

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We aimed to map the probability of *Schistosoma haematobium* infection being >50%, a threshold for annual mass praziquantel distribution. Parasitologic surveys were conducted in Burkina Faso, Mali, and Niger, 2004–2006, and predictions were made by using Bayesian geostatistical models. Clusters with >50% probability of having >50% prevalence were delineated in each country.

Large-scale control programs for tropical infectious diseases have been initiated in recent years (*1*,*2*), after renewed commitment by governments and international funding agencies to support the control of previously neglected tropical diseases, including parasitic diseases such as malaria, schistosomiasis, onchocerciasis, lymphatic filariasis, and soil-transmitted helminth infections. Schistosomiasis is the second-most important parasitic disease throughout the world, with an estimated 207 million persons infected (*3*).

Success and sustainability of large-scale disease control programs depend on the allocation of resources where they will have maximum benefit (*4*). Given that tropical infectious diseases, such as schistosomiasis, tend to occur in spatially defined foci (i.e., clusters or hot spots) (*5*), efficient resource allocation relies on identifying the location of high-risk populations. Because disease-endemic countries do not have sophisticated surveillance systems that can accurately delineate disease clusters, alternative methods such as sample-based spatial prediction need to be applied to target control programs.

Among the multinational and multi-institutional partnerships formed to confront the problem of neglected tropical diseases is the Schistosomiasis Control Initiative (SCI; www.schisto.org), which supports national schistosomiasis and soil-transmitted helminth control programs in Burkina Faso, Mali, and Niger (and other African countries). Lengeler et al. (*6*) describe 2 approaches to targeting interventions: one in which the number of recipient schools or communities is determined by available resources and the other in which a prevalence threshold is defined above which all schools or communities benefit from the intervention. SCI takes the latter approach, delineating areas according to the World Health Organization (WHO)–recommended threshold prevalence of 50% for annual mass treatment. However, even this approach needs to take into account factors such as resource availability and decision risk because uncertainties exist when delineating areas based on the selected threshold.

Knowledge of uncertainty regarding the location and spatial dimensions of clusters is important because it makes possible a prior assessment of the risks and potential consequences associated with different resource allocation strategies. Uncertainties in spatial prediction maps originate from factors such as natural random variation and measurement error of the outcome variable and covariates. Bayesian methods are useful because they provide an approach for propagating uncertainty (through a prediction model) in regards to the spatial predictions. Only recently have practical applications of Bayesian methods in large-scale tropical disease control programs been reported (*7*–*9*).

The Study

The objective of this study was to produce maps that could be integrated into the SCI-supported national intervention strategies and that explicitly represent uncertainties in spatial predictions so that national control managers could judge the quality of the evidence upon which the strategies will be based. The SCI-supported programs involve mass distribution of praziquantel (for urinary and intestinal schistosomiasis) and albendazole (for soil-transmitted helminths). The parasitic infection with the highest prevalence is urinary schistosomiasis, caused by flukes (*Schistosoma hematobium*), and the programs are planned to control this disease (*2*).

Parasitologic data were collected in coordinated school-based field surveys in Burkina Faso, Mali, and Niger (Figure 1) during 2004–2006 (preintervention) by using standardized protocols (available on request). The collated dataset covered a spatially contiguous area, ≈2,750 km × 850 km, and included the infection status of 27,939 school-age children in 418 randomly selected locations. Infection

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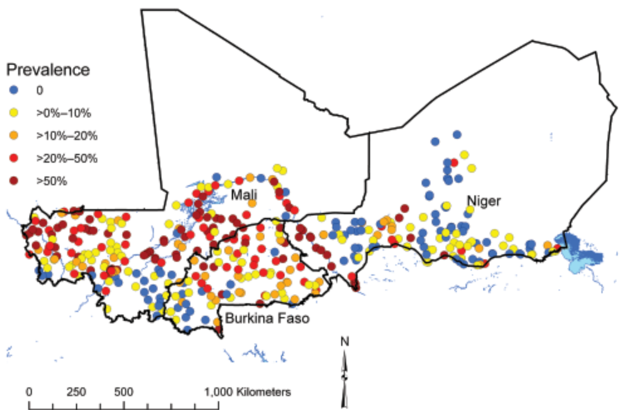

Figure 1. Prevalence of infection with *Schistosoma hematobium* at 418 survey locations in Burkina Faso, Mali, and Niger, 2004–2006.

status was defined according to egg count determined by microscopic examination of urine samples; ≥1 *S. hematobium* eggs indicated infection.

Spatial prediction was based on a logistic regression model (Table), constructed by using the software WinBUGS, version 1.4.2 (MRC Biostatistics Unit, Cambridge and Imperial College, London, UK). The model had infection status as the binary outcome variable, age and sex of the survey participants as individual-level fixed effects, and distance from perennial water body (derived from electronic maps obtained from the Food and Agriculture Organization) and land surface temperature (LST; with a quadratic term; see Hay et al. [*10*] for details on how these data were derived) as survey location–level fixed effects. Variable selection methods and the model are presented in the online Technical Appendix (available from www.cdc.gov/EID/content/14/10/1629-Techapp.pdf). The model also included a geostatistical random effect for residual spatial clustering of infection prevalence (*11*).

A prevalence map for the study area was constructed, using the model, by predicting infection prevalence at the centroids of cells of a 0.15 × 0.15 decimal degree (≈18 km × 18 km) grid. This model was implemented with the *spatial.unipred* command of WinBUGS (details are provided in the online Technical Appendix). Estimates from Bayesian models are distributions (termed posterior distributions) that represent the probability of each of a range of plausible values being true for the variable being modeled. To quantify the uncertainties surrounding the model predictions, we plotted the probability of each prediction location having a prevalence >50%, rather than mean predicted prevalence at each location. The probabilities were calculated from the posterior distributions of predicted prevalence at each location (i.e., if 95% of the posterior distribution of predicted prevalence was >0.5, the probability of prevalence >50% at that location was 95%).

Cross-validation was done by randomly allocating survey locations to 3 groups and undertaking 3 separate runs of the model; 1 of the 3 groups was sequentially omitted, and predicted prevalence at the omitted locations was determined by using the model. Predicted prevalence was compared with observed prevalence, dichotomized, according to a 50% observed prevalence threshold. The comparison statistic was the area under the curve (AUC) of the receiver operating characteristic, and a value of >0.7 was considered to indicate acceptable predictive ability. An average AUC was calculated across the 3 model runs.

In the final model (Table), statistically significant correlations suggested that infection prevalence was higher in older boys and increased with proximity to perennial bodies of water, but no association was found between prevalence and LST. The range over which spatial correlation was >5% (chosen to indicate statistically important spatial correlation) was ≈177 km, indicating the approximate radius of clusters. Results of the validation analysis showed an average AUC of 0.86, indicating that the model had an acceptable predictive performance.

Bayesian probability maps were produced for each sex and age group, but for illustrative purposes we present predicted probability of prevalence >50% in boys ages 13–16 years (the group with the highest infection prevalence; Figure 2). Large clusters of prediction locations with a high probability (i.e., >50%; indicative of low uncertainty) of

Table. Bayesian logistic regression model of prevalence of infection with *Schistosoma haematobium* in children in 418 schools in Burkina Faso, Mali, and Niger, 2004–2006*

Variable	Posterior distribution	
	Mean (95% CrI)	SD
Female gender	0.70 (0.65–0.76)	0.03
Age, y		
9–10	1.16 (1.00–1.33)	0.08
11–12	1.51 (1.31–1.73)	0.10
13–16	1.79 (1.53–2.06)	0.14
Distance to perennial water body	0.34 (0.21–0.54)	0.08
Land surface temperature	0.80 (0.51–1.21)	0.18
Land surface temperature^2	1.10 (0.85–1.40)	0.14
Rate of decay of spatial correlation	2.03 (1.48–2.74)	0.32
Variance of the spatial random effect (sill)	7.03 (5.36–9.31)	1.01

*CrI, Bayesian credible interval. Values for the fixed effects are odds ratios; note the odds ratios for the climate variables are on a common scale, where the variables were standardized to have a mean = 0 and SD = 1. The reference group for sex was boys and for age was 6–8 y. The number of children found to be infected with *S. haematobium* was modeled by using a binomial distribution described by the proportion infected and the total number sampled in each survey location. The proportion infected was modeled by using logistic regression with an intercept, covariates (sex, age, distance to perennial water body, land surface temperature, and a quadratic term for land surface temperature), and a random effect that described spatial correlation (i.e., clustering). Model outputs were distributions (termed *posterior distributions*) that can be summarized by using the mean, SD, and 95% CrI (representing the range of values that contains the true value with a probability of 95%). More details on the model are presented in the online Technical Appendix (available from www.cdc.gov/EID/content/14/10/1629-Techapp.pdf).

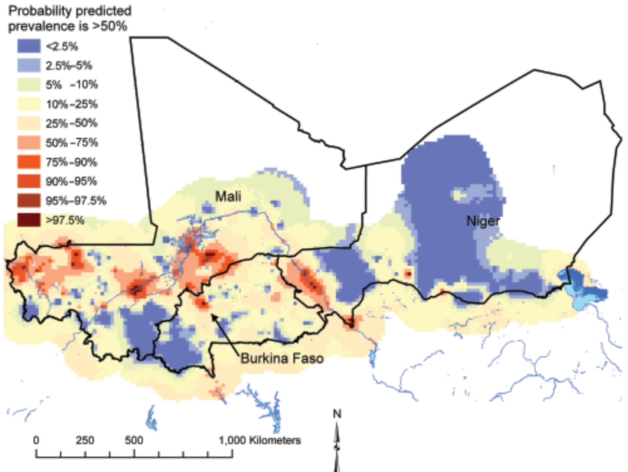

Figure 2. Predicted probability of prevalence of infection with *Schistosoma hematobium* being >50% in Burkina Faso, Mali, and Niger in boys ages 13–16 years; results are based on a Bayesian geostatistical model. The red areas had a low degree of uncertainty that predicted prevalence was >50%, and the blue areas had a high degree of uncertainty that predicted prevalence was >50%.

prevalence being >50% intervention threshold were located in a mid-latitudinal band across Mali, running from western to central regions, and in the Niger River valley region of Niger. Smaller clusters were located in various parts of southern and eastern Mali, northwestern and northeastern Burkina Faso, and south-central Niger.

Conclusions

Future schistosomiasis control plans should acknowledge uncertainties such as those presented in Figure 2. A possible approach would be to introduce a second threshold for the level of uncertainty that a location is above the intervention prevalence threshold; if the uncertainty is greater than this second threshold, then the location is excluded until new evidence is obtained that confirms prevalence is above or below the intervention prevalence threshold. This second uncertainty threshold should be determined by the quantity of resources available for disease control and the level of decision risk deemed appropriate.

In addition to providing an evidence base for distributing resources in 3 West African countries as part of the SCI-supported national control programs, the maps presented here have a potential role in maintaining sustainability of schistosomiasis control after SCI support ends (SCI is funded through 2009). They can be used as advocacy tools for channeling funds to high-risk populations in the affected countries and, in the likely event that money for schistosomiasis control in these countries becomes more limited after SCI support ends, they can be used to ensure that scarce governmental resources are distributed as efficiently as possible. National coordinators who might face accountability for targeted (i.e., unequal) distribution of resources will benefit from the defendable, scientifically sound methods presented in this article. By focusing on uncertainty in spatial predictions, more flexible tools for disease control can be developed that allow the geographic dimensions of control programs to be scaled and modified according to available resources and acceptable levels of decision risk.

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Dr Clements is an infectious disease epidemiologist with a particular interest in the spatial epidemiology of tropical infectious diseases. A major focus of his research is implementation of Bayesian spatial modeling and prediction to enhance planning of real-world, large-scale tropical infectious disease control programs.

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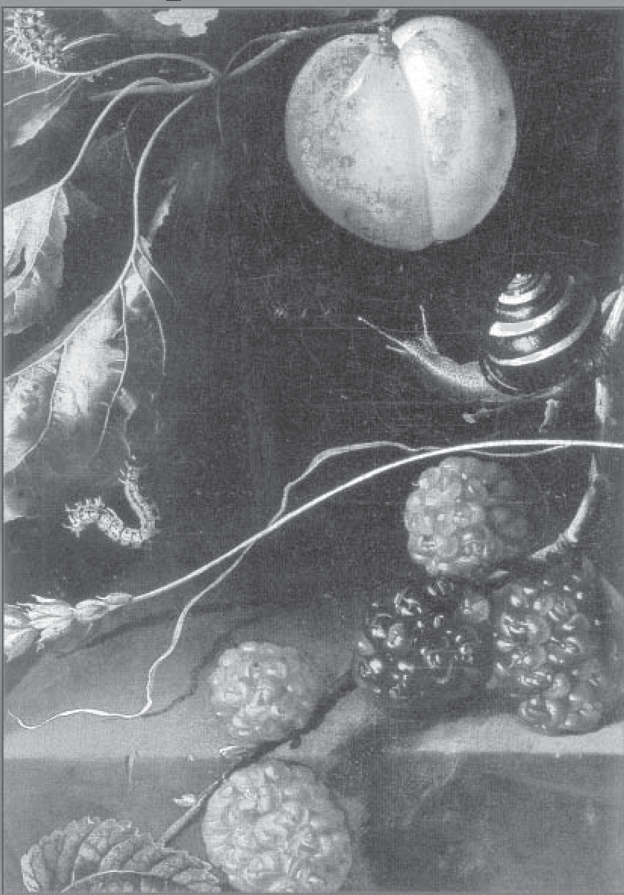

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Pathogen Emergence

Host Susceptibility

Chronic Sequelae

Risk Assessment

Consumer Concern

Spinach-associated *Escherichia coli* O157:H7 Outbreak, Utah and New Mexico, 2006

Juliana Grant,[1] Aaron M. Wendelboe,[2] Arthur Wendel, Barbara Jepson, Paul Torres, Chad Smelser, and Robert T. Rolfs

In 2006, Utah and New Mexico health departments investigated a multistate cluster of *Escherichia coli* O157:H7. A case–control study of 22 case-patients found that consuming bagged spinach was significantly associated with illness (p<0.01). The outbreak strain was isolated from 3 bags of 1 brand of spinach. Nationally, 205 persons were ill with the outbreak strain.

On September 13, 2006, health officials from several states independently notified the Centers for Disease Control and Prevention (CDC) about clusters of *Escherichia coli* O157:H7 infections and a suspected association with spinach. *E. coli* O157:H7 expresses 1 of 2 types of Shiga toxin and can cause severe gastrointestinal infections and hemolytic uremic syndrome (HUS).

A multistate outbreak investigation, involving 26 states, was initiated on September 14. The US Food and Drug Administration (FDA) and CDC advised consumers not to eat bagged spinach (*1,2*). The Utah (UDOH) and New Mexico Departments of Health conducted a case-control study to characterize the outbreak and a laboratory investigation to test spinach eaten by case-patients for contamination. This report focuses on the investigation conducted in the 2 states.

The case definition for a laboratory-confirmed illness was culture-confirmed *E. coli* O157:H7 infection in a Utah or New Mexico resident with illness onset during August 1, 2006–October 1, 2006, and a pattern of Xba EXHX01.0124 shown by pulsed-field gel electrophoresis (PFGE). This urgent outbreak investigation did not require institutional review board approval.

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The Study

The UDOH Enteric Disease Case Report Form and a supplemental CDC questionnaire on spinach consumption were administered by local or state public health officials to all participants by telephone. Information collected included date of disease onset, symptoms, treatment, community-based exposures, and a food-item history. Questions referred to the 8–10 days before case-patient symptom onset. Case-patients were first interviewed 3–23 days after illness onset (mean = 11.6 days); follow-up interviews for the questionnaire were completed within 23 days of illness onset.

Two controls per case-patient were matched by sex and age group to prevent confounding from potential differences in diet (*3*); age groups were ≤4 years, 5–12 years, 13–18 years, 19–64 years, and ≥65 years. Controls were selected by using sequential-digit telephone dialing based on the matched case-patient's telephone number. Controls reported no gastrointestinal illness 3 days before and after symptom onset date of their matched case-patient.

Exact matched odds ratios (mOR) and confidence intervals (CI) were calculated by using conditional logistic regression in SAS 9.1 (SAS Institute, Cary, NC, USA). An α of 0.05 was used. No statistical analyses were performed for the categories "spinach brand" or "location spinach was eaten" because of insufficient data and inability to generate point estimates; we provide only descriptive evaluations of these variables. Only persons who indicated definite exposure to a single brand of spinach were included in the evaluation of brand.

The Utah Public Health Laboratories (UPHL) and New Mexico Scientific Laboratory Division (NMSLD) provided analytic testing services for all clinical and spinach samples. Public health officials collected spinach samples in their original packaging from confirmed case-patients. One spinach sample in New Mexico was frozen; all other samples were refrigerated. Modified FDA Bacterial and Analytical Manual methods were used to recover *E. coli* O157:H7 from both clinical and spinach samples (*4*). UPHL used MacConkey broth and NMSLD used Food Emergency Response Network (FERN) broth for enrichment instead of Enterohemorrhagic *E. coli* Enrichment Broth; both laboratories used an additional selective media (CHROMagar, CHROMagar, Paris, France).

The presence of O157 and H7 antigens in clinical and spinach samples was confirmed with latex agglutination typing. UPHL used ProLexLatex Agglutination System (Pro-Lab Diagnostics, Austin, TX, USA); NMSLD used Oxoid Dryspot *E. coli* O157 test kit (Oxoid, Cambridge,

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UK) and DIFCO H7 antiserum (DIFCO Laboratories, Detroit, MI, USA). All clinical isolates were tested for Shiga toxin genes (*stx1* and *stx2*) by PCR. Clinical and spinach isolates were tested for Shiga toxin expression with the Premier EHEC enzymatic immunoassay Shiga-toxin test kit (Meridian Diagnostics, Inc., Cincinnati, OH, USA). PFGE was performed on *E. coli* O157:H7 cultures from clinical and spinach samples by using standard CDC PulseNet operating procedures (*5*). PCR testing reagents and protocols were provided through the Laboratory Response Network (LRN) and FERN.

Eighteen cases were confirmed in Utah and 5 in New Mexico. Onset dates ranged from August 22 to September 11 (Figure). Shiga toxin 2 was detected in stool samples from all patients. Demographic, clinical, and food exposure information was available for all patients (Table 1, 2). Fifty-seven percent of case-patients were hospitalized, and 29% experienced HUS (age range 2–60 years). No deaths were reported.

Matched analyses were performed on 22 case-patients and 44 matched controls. Consumption of bagged spinach (mOR = 18.7, 95% CI = 2.8–797.1, p<0.01) was significantly associated with case status (Table 2). Food items previously implicated as sources of pathogenic *E. coli* were not significantly associated with case status.

No patients, versus 6 controls, reported only eating spinach at a restaurant; 16 patients and 10 controls reported only eating spinach in a private home (Table 2). Washing spinach before eating did not significantly change the odds of being a case-patient (Table 2). No study participants reported eating only cooked spinach; therefore, cooking was not analyzed. Ten case-patients and 3 controls reported definite exposure to single brands of spinach; only brands A and B were reportedly consumed by these patients. Seven case-patients and 1 control reported definite exposure to brand A; 0 case-patients and 2 controls reported definite exposure to brand B (Table 2).

Seven bags of spinach (5 from Utah, 2 from New Mexico) were provided by 7 case-patients. Five bags had been opened and their contents partially consumed. *E. coli* O157:H7, matching the national outbreak strain, was detected by PCR and culture in 2 Utah spinach bags and the New Mexico unfrozen bag. All 3 were open bags of brand A baby spinach. Lot codes were available on 2 bags; both were packed on August 15, 2006, at the same plant, on the same shift, but on different machines.

The 4 bags from which *E. coli* O157:H7 was not detected were also brand A baby spinach and were packed on August 15, 22, 23, and 28, 2006. Two bags that tested negative were eaten by case-patients who reported eating from multiple bags before illness onset.

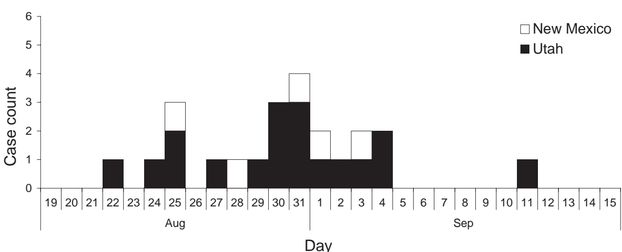

Figure. Epidemic curve by date of onset of confirmed cases, *Escherichia coli* O157:H7 spinach-associated outbreak, Utah and New Mexico, 2006.

Conclusions

Consumption of fresh brand A spinach was associated with *E. coli* O157:H7 infection with both epidemiologic and laboratory data. Washing spinach before consumption did not affect odds of being a case-patient. Possible reasons for this include 1) *E. coli* could be internalized into the plant structure by entering through the roots (*6*), and 2) bacteria are more likely to adhere to cut surfaces of leafy greens (e.g., prepackaged spinach) (*7*). That no case-patients reported only eating spinach in a restaurant suggests that the source of the contaminated spinach did not supply commercial establishments.

The percentage of case-patients in whom HUS developed (29%) was high when compared with that in prior *E. coli* O157:H7 outbreaks (15%–20%) (*8*,*9*). This finding is consistent with studies that associate Shiga toxin 2–expressing *E. coli* with a higher incidence of HUS (*10*,*11*).

Our study was subject to certain limitations. One is potential recall bias since controls had a longer time lag between consumption and interview and less motivation

Table 1. Demographic and clinical information on confirmed cases, *Escherichia coli* O157:H7 spinach-associated outbreak, Utah and New Mexico, 2006

Characteristic	No. (%), n = 23
Sex	
M	5 (22)
F	18 (78)
Age group, y	
≤4	4 (17)
5–12	5 (22)
13–18	1 (4)
19–64	12 (52)
≥65	1 (4)
State of residence	
New Mexico	5 (22)
Utah	18 (78)
Clinical outcome	
Bloody diarrhea	22 (96)
Hospitalized	13 (57)
Hemolytic uremic syndrome	7 (29)

Table 2. Bivariate analysis of statistically significant spinach-related exposures during 8–10 d before onset, *Escherichia coli* O157:H7 spinach-associated outbreak, Utah and New Mexico, 2006*

Variable	Case-patients	Controls	Matched OR	95% CI	p value
Ate any spinach					
Yes	18	15	16.5	2.4–710.2	0.0005
No	3	28	1.0		
Ate bagged spinach					
Yes	17	8	18.7	2.8–797.1	0.0001
Other type of spinach	1	2	10.1	0.1–988.8	0.41
No spinach	3	28	1.0		
No. of times ate spinach					
>2	8	2	†	3.6–∞	0.0004
1–2	7	9	7.4	0.8–354.7	0.08
0	3	28	1.0		
Rewashed spinach					
Yes	5	4	1.0	0.08–13.8	1.0
No	9	6	1.0		
Location where spinach eaten‡					
Restaurant	0	6			
Private home	16	10			
Brand					
Ate only brand A	7	1			
Ate only brand B	0	2			

*OR, odds ratio; CI, confidence interval.
†Unable to calculate a maximum likelihood estimate for a matched OR.
‡Certain persons reported having eaten spinach both at home and in restaurants.

to accurately recall what foods they had eaten. However, overestimation of the association between spinach consumption and illness is unlikely because more controls reported having eaten spinach than were previously identified in surveys of the general population (*12*). Analysis of brand was limited because of poor recall among all participants. The sample size was small, resulting in imprecise effect estimates and, in certain cases, an inability to calculate a measure of association. Exact ORs were used to partially counteract this limitation.

This investigation was conducted in response to a national outbreak of *E. coli* O157:H7 infections with matching PFGE patterns among 205 persons in 26 states (*13*). Less than 2 weeks after its initiation, this investigation provided laboratory and epidemiologic evidence implicating spinach. The FDA used these data to focus its field investigation and interventions and linked the contaminated spinach to samples taken from a stream, cattle manure, and feces from wild pigs on ranches in Salinas Valley, California (*13*). In August 2006, FDA launched a lettuce safety initiative to address recurring outbreaks of *E. coli* O157 infections (*14*). After this outbreak, the initiative was expanded to include all leafy greens.

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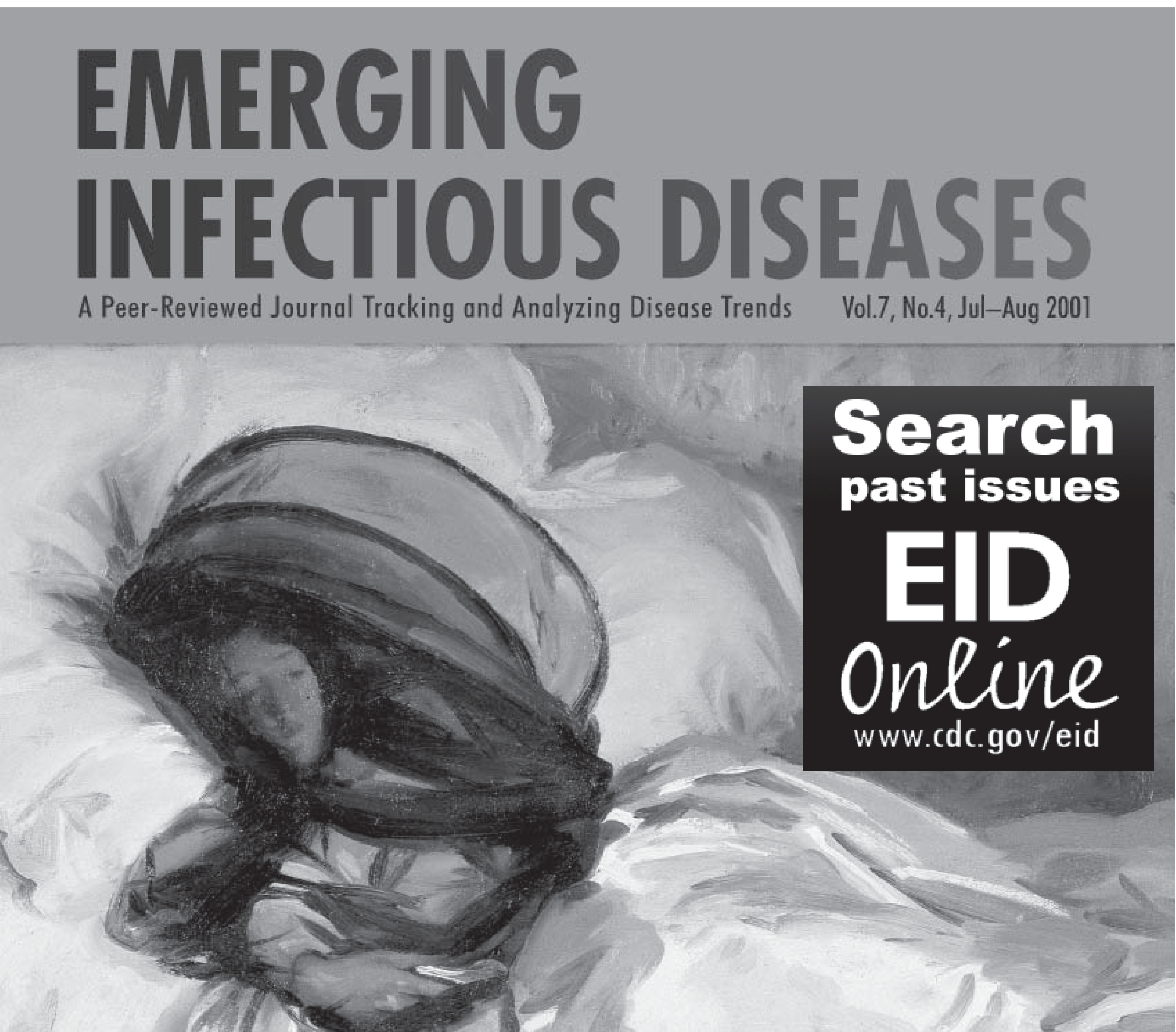

Molecular Surveillance for Multidrug-Resistant *Plasmodium falciparum*, Cambodia

Naman K. Shah, Alisa P. Alker, Rithy Sem, Agustina Ika Susanti, Sinuon Muth, Jason D. Maguire, Socheat Duong, Frederic Ariey, Steven R. Meshnick, and Chansuda Wongsrichanalai

We conducted surveillance for multidrug-resistant *Plasmodium falciparum* in Cambodia during 2004–2006 by assessing molecular changes in *pfmdr1*. The high prevalence of isolates with multiple *pfmdr1* copies found in western Cambodia near the Thai border, where artesunate–mefloquine therapy failures occur, contrasts with isolates from eastern Cambodia, where this combination therapy remains highly effective.

The Thailand–Cambodia border has been an epicenter for drug-resistant *Plasmodium falciparum*. Recent clinical studies indicate that efficacy of artesunate–mefloquine combination is decreasing on both sides of the border (*1–3*). In contrast, *P. falciparum* in eastern Cambodia remains sensitive to mefloquine and the artesunate–mefloquine combination (*4*,*5*). Declining artesunate–mefloquine efficacy on the Thai border and the geographic variation in susceptibility to this combination therapy suggest expanded surveillance is needed in Cambodia. An inexpensive method to help target in vivo monitoring sites is surveillance for molecular markers of drug resistance.

We have previously shown that elevated *P. falciparum* multidrug resistance 1 (*pfmdr1*) gene copy number is associated with an 8-fold risk for artesunate–mefloquine failure in western Cambodia (*6*). More recently, we conducted a molecular surveillance for *pfmdr1* mutations to guide the selection of sentinel sites for in vivo monitoring of artesunate–mefloquine resistance.

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The Study

Clinical isolates of *P. falciparum* were collected from 5 sites across Cambodia (Figure 1): Pailin, Kampong Seila, Chumkiri (western), Memut, and Rattanakiri (eastern) during 2004–2006. Study participants included patients who were seen at health centers with uncomplicated falciparum malaria, including mixed infections. Fifty-six samples from patients in Pailin had been previously analyzed (*6*). Institutional review board approvals were obtained from the Cambodian National Ethics Committee for Health Research, the US Naval Medical Research Unit No. 2, and the University of North Carolina at Chapel Hill (UNC).

Peripheral blood was used to prepare smears and filter paper blood spots (25 µL, 2 spots) on Whatman 3-mm blotting paper (Whatman International Ltd., Maidstone, Kent, UK) from each participant. Blood smears were examined to determine parasite density, nonfalciparum species, and gametocytes. Enrolled participants completed a questionnaire on demographic information and past medical history. They received artesunate–mefloquine in accordance with national guidelines for uncomplicated falciparum malaria. DNA was extracted from blood spots by using QIAmp Mini kits (QIAGEN, Valencia, CA, USA). *pfmdr1* copy number and single-nucleotide polymorphism assays were performed as previously described (*6*,*7*), except that in *pfmdr1* single-nucleotide polymorphism assays the concentration of probes was reduced from 250 nM to 125 nM and reactions were not duplicated.

Linear regression was used to determine if *pfmdr1* copy number varied by site. *pfmdr1* copy number was in-

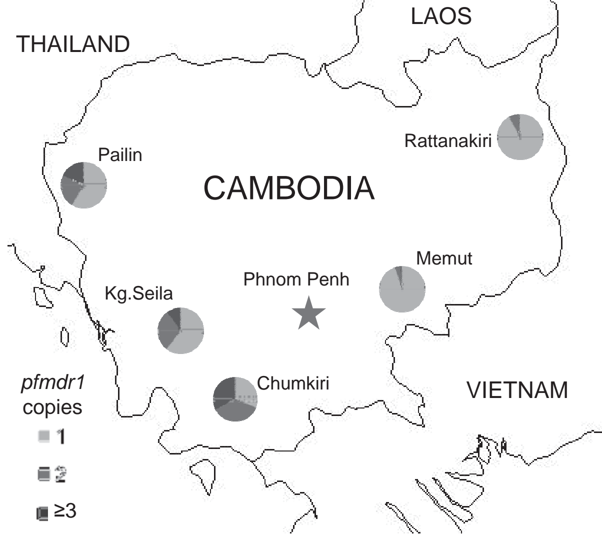

Figure 1. Map of Cambodia with locations of surveillance sites and proportion of isolates containing 1, 2, or ≥3 copies of *pfmdr1*, May 2004–December 2006. Kg., Kampong.

verse transformed to meet assumptions of normality and homoscedasticity. Backwards elimination based on a p value of 0.05 was used to determine the effect of site when controlling for confounders. Logistical regression was used to confirm the results of the linear regression analysis by using a cutoff of 1.5 copies (*8*). Logistic regression was also used to determine if *pfmdr1*-184 genotype varied by site. Samples with a mixture of 184-phe and 184-tyr were coded as 184-phe. All statistical analyses were conducted in Stata 8.2 (StataCorp, College Station, TX, USA).

We enrolled 744 study participants with uncomplicated *P. falciparum* malaria. The characteristics of participants at each site are described in Table 1. *pfmdr1* copy number was successfully determined for 712 (95.7%) of 744 samples. When compared with results at UNC, results of copy number assays performed by the National Malaria Control Program (NMCP) staff in Phnom Penh were slightly lower (mean difference –0.163, 95% confidence interval [CI] –0.277 to –0.049, n = 44), and the 2 groups were in 100% concordance with distinguishing samples with >2 *pfmdr1* copies.

pfmdr1 copy numbers ranged from 0.6 to 6.3, and 167 (23.5%) of 712 samples had >1.5 copies. The median and distribution of *pfmdr1* copy number varied by site and were highest in the 3 western Cambodian sites (Figure 2). The proportion of samples with >1.5 *pfmdr1* copies was greater in western than eastern Cambodia (52.7% vs. 6.4%, odds ratio [OR] = 16.2, 95% CI 10.3–25.3). The prevalence of parasite samples with amplified *pfmdr1* varied by site and differed in the prevalence of 2 and ≥3 copies of *pfmdr1* (Figure 1).

The prevalence of *pfmdr1*-184-Phe varied by site, was significantly higher in the West (85.5% vs. 17.1%, OR = 27.4, 95% CI 18.1–41.4), but was not more prevalent in samples with *pfmdr1* copy number >1.5 when site was controlled for (OR = 1.5, 95% CI 0.9–28, p = 0.139). *pfmdr1* codons 86, 1034, and 1042 were predominantly wild-type, 356 (98.6%) of 361, 215 (97.7%) of 220, 347 (95.9%) of 362, respectively, with little variation between sites (data not shown). Most (196 [91.2%] of 215) samples had the haplotypes 86-Asn, 1034-Ser, and 1042-Asn.

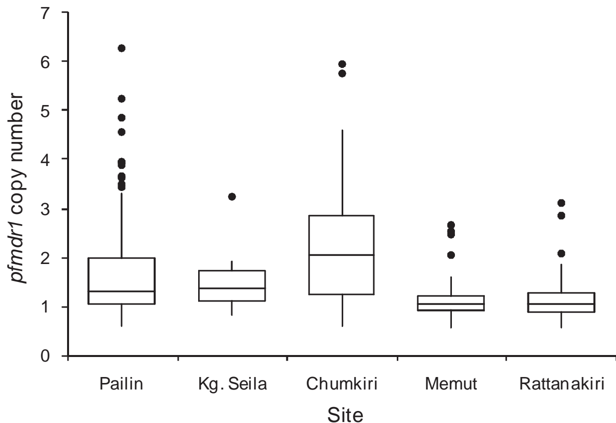

Figure 2. Median values and interquartile ranges of *pfmdr1* copy number across 5 surveillance sites in Cambodia, May 2004–December 2006. Kg., Kampong.

In univariate and multivariate linear regression models, site was the strongest predictor of *pfmdr1* copy number. Chumkiri had the highest *pfmdr1* copy number before and after clinical correlates were controlled for (Table 2). The relationship between copy number and site was retained when the analysis was repeated with logistic regression. Compared with Rattanakiri, the OR of having a copy number >1.5 was 32.4 (95% CI 16.4–64.2) for Chumkiri, 7.8 (95% CI 4.3–13.9) for Pailin, and 0.5 (95% CI 0.2–1.2) for Memut when parasitemia and year were controlled for.

Conclusions

We found geographic heterogeneity for 2 *pfmdr1* genetic markers: *pfmdr1* 184 and *pfmdr1* amplification. Genotype 184-Phe was associated with *pfmdr1* amplification at the population level but not in individual isolates. The basis of this trend is unknown. Pailin, Kampong Seila, and Chumkiri in western Cambodia had higher *pfmdr1* copy numbers and higher prevalence of *pfmdr1* 184-Phe

Table 1. Clinical characteristics of study participants at 5 surveillance sites, Cambodia, May 2004–December 2006*

Characteristic	Pailin	Kg. Seila†	Chumkiri	Memut	Rattanakiri
Total no.	146	11	116	172	299
Male, no. (%)	98 (67.1)	9 (81.8)	97 (83.6)	62 (36.1)	187 (62.5)
Adult (≥18 y), no. (%)	128 (87.7)	11 (100.0)	109 (94.0)	172 (100.0)	221 (73.9)
Parasitemia,‡ geometric mean	15,001	5,975	13,503	19,800	22,443
Parasitemia >50,000, no. (%)	47 (32.2)	4 (36.4)	33 (28.7)	68 (39.8)	120 (40.4)
Gametocytemia, no. (%)	8 (5.5)	1 (9.0)	11 (9.6)	12 (7.0)	8 (2.7)
Smear-positive malaria in past y, no. (%)	35 (39.3)	2 (20.0)	25 (61.0)	13 (7.6)	127 (46.7)
Antimalarial drug taken in past mo, no. (%)	13 (14.4)	2 (18.2)	20 (17.2)	64 (37.2)	6 (2.0)
Mixed infection, no. (%)	6 (4.1)	1 (9.1)	8 (7.0)	3 (1.8)	6 (2.0)
No. d of illness, median (range)	3 (1–6)	4 (2–6)	6 (1–6)	4 (2–6)	4 (0–6)

*The missing values by category include parasitemia (n = 4), gametocytemia (n = 4), smear-positive malaria in past year (n = 160), antimalarial drug taken in past month (n = 56), mixed infection (n = 4), and no. of d of illness (n = 21).
†Baseline differences were most likely due to small sample size.
‡Parasites/μL.

Table 2. Univariate and multivariate linear regression analyses of the association between surveillance site and *pfmdr1* copy no., Cambodia

Site	No.	Mean	Univariate linear regression		Multivariate linear regression*		
			β†	95% CI‡	Predicted mean	β†	95% CI‡
Rattanakiri	285	1.09	0		1.03	0	
Memut	165	1.09	–0.001	–0.056 to 0.053	1.02	0.047	–0.019 to 0.112
Kampong Seila	10	1.53	–0.234	–0.412 to –0.055	1.35	–0.184	–0.363 to –0.006
Pailin	143	1.70	–0.232	–0.289 to–0.175	1.34	–0.217	–0.276 to –0.159
Chumkiri	109	2.24	–0.393	–0.456 to –0.331	1.72	–0.432	–0.503 to –0.362

*Controlled for parasitemia and year.
†The coefficient estimating the relationship between site and inverse-transformed *pfmdr1* copy no.
‡CI, confidence interval.

than Memut and Rattanakiri in eastern Cambodia. An association between these markers and resistance is consistent with reports of reduced efficacy of artesunate–mefloquine in Pailin (79% over 42 days) (*2*) compared with Memut and Rattanakiri, where artesunate–mefloquine efficacy remains close to 100% (*4*,*5*). This finding is also consistent with the decrease of mefloquine sensitivity in western Cambodia based on in vitro drug susceptibility monitoring conducted by the Pasteur Institute of Cambodia from 2001 through 2007 (*9,10*; P. Lim, pers. comm.).

Chumkiri was surveyed because local health staff observed that falciparum malaria patients treated with artesunate–mefloquine often returned within weeks with recurrent fever and parasitemia. The possibility of such resistance in this site was particularly alarming because it was thought to be confined to Thailand–Cambodia border areas. Chumkiri had not previously been a sentinel site for antimalarial efficacy monitoring by NMCP. On the basis of these *pfmdr1* data, a clinical trial of artesunate–mefloquine was launched in Chumkiri in 2006 (*11*). Clinical validation of predicted resistance by in vivo studies is beyond the scope of this study, although multiple in vitro and in vivo studies have consistently shown an association of increased *pfmdr1* copy number with mefloquine or artesunate–mefloquine failure in Southeast Asia (*8*,*10*,*12–15*), supporting use of *pfmdr1* copy number in routine surveillance and policy formation.

Monitoring changes in antimalarial drug efficacies is essential for guiding treatment policies in an era of multidrug resistance. However, such studies are resource intensive. In Cambodia, for example, NMCP can conduct in vivo studies at only 2–3 sites per year because of limited funds and trained staff. Molecular markers can help target in vivo studies where they are most needed. Molecular surveillance is high-throughput and can be performed in a central laboratory on dried blood spots. Expanded surveillance allows for molecular mapping and enables a rapid containment of resistance foci. *pfmdr1* assays are now routinely performed in Cambodia by NMCP staff. National and regional molecular surveillance by malaria-endemic countries is a real possibility.

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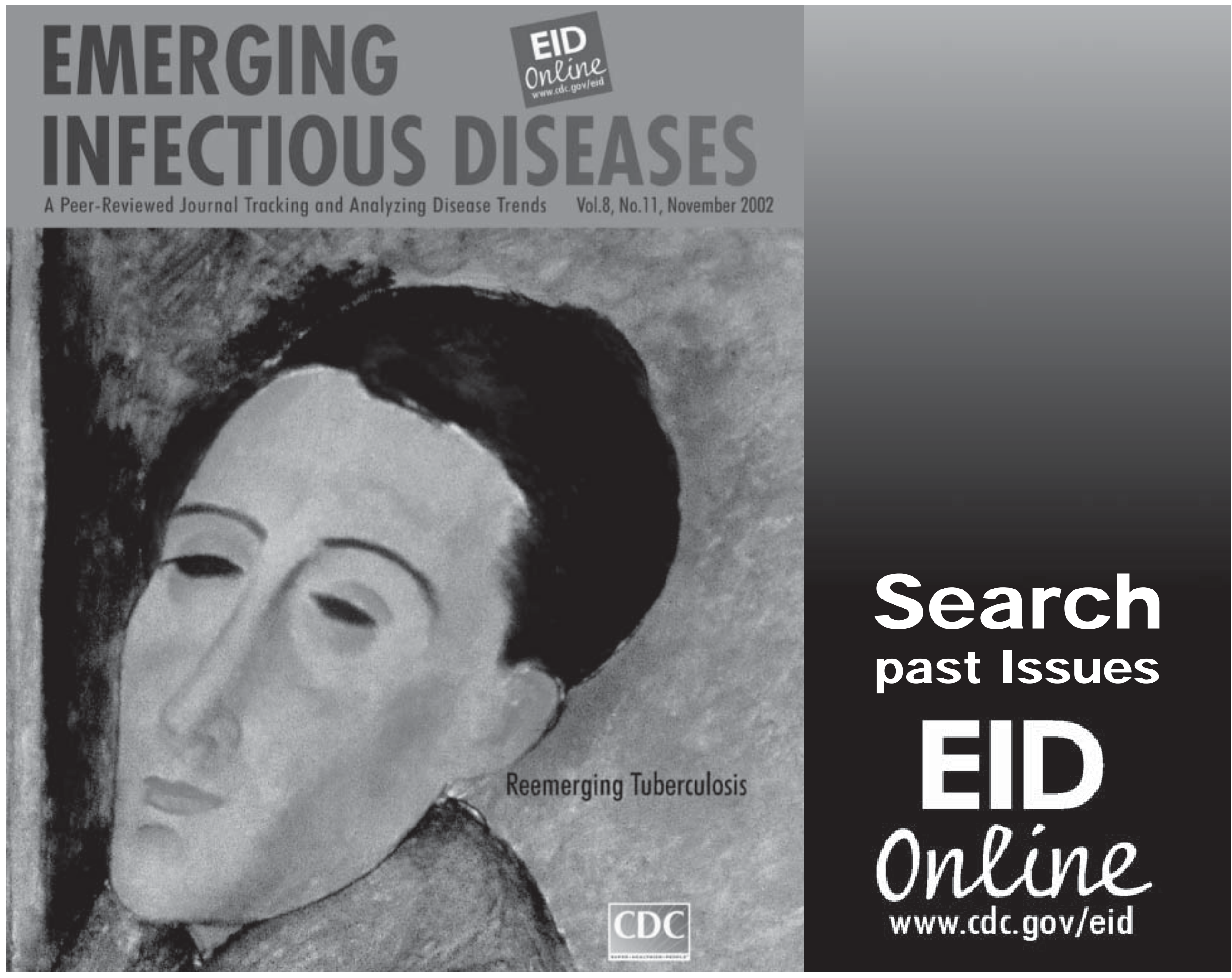

Decreased Tuberculosis Incidence and Declining Clustered Case Rates, Madrid

Jesús Iñigo, Araceli Arce, Elia Palenque, Darío García de Viedma, and Fernando Chaves

To determine effect of recent tuberculosis transmission rates on incidence rates, we conducted 2 prospective population-based molecular epidemiologic studies in Madrid during 1997–1999 (4% immigrants) and 2002–2004 (14.9% immigrants). Case rates decreased in association with declining clustered case rates among Spanish-born persons. New strains were introduced through immigration.

During the past decade in Madrid, Spain, tuberculosis (TB) case rates have decreased substantially while the proportion of foreign-born persons with TB has increased (2.6% in 1994 to 33.7% in 2003) (*1*). We used a combination of genotyping and conventional epidemiologic investigation to determine the extent to which the decline in incidence of TB in Madrid was affected by changes in rate of recent transmission of disease.

The Study

We conducted 2 prospective population-based studies of TB patients in 3 urban districts of Madrid over 2 separate periods: 1997–1999 (population 455,050; 4% immigrants) and 2002–2004 (population 488,518; 14.9% immigrants). We included all TB patients in these 3 districts and used the same methods for both studies. All clinical samples were sent to the Microbiology Department of Hospital 12 de Octubre for TB testing. All patient information was collected by using a standardized protocol based on the Regional Registry of Tuberculosis Cases in Madrid.

Clinical specimens were processed according to standard methods. DNA fingerprinting was performed by restriction fragment length polymorphism (RFLP) analysis with the insertion sequence IS*6110*. Computer-assisted analysis was performed by using Bionumerics software (Applied Maths, Kortrijk, Belgium). All strains with <6 copies of IS*6110* were spoligotyped. Within each study period, cases were considered to be clustered if common RFLP patterns containing ≥6 indistinguishable IS*6110* bands or patterns containing ≤5 indistinguishable IS*6110* bands and identical spoligotyping patterns were found.

For each period we calculated average annual TB case rates and rates of clustered and nonclustered cases. Census populations of each district were used as denominators. Statistical analyses used Epi Info version 3.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and Epidat (Pan American Health Organization, Washington, DC, USA).

Case numbers were 412 during 1997–1999 (average incidence 30.2 cases/100,000) and 377 during 2002–2004 (average incidence 25.7/100,000); p<0.001 (Table 1). The 150 (19%) foreign-born patients were from 20 different countries. Date of arrival in Spain was available for 81 (54%); 78 (96.3%) had been in Spain <5 years before TB diagnosis, and 39 (48.2%) diagnoses were made within the first 2 years of residence. Median time from arrival to onset of treatment was 30.1 months (25th–75th percentiles 8.0–50.7).

In the first period, 328 cases (79.6%) were confirmed by isolation of *Mycobacterium tuberculosis*. RFLP analysis was performed on 212 isolates (64.6% of culture-positive cases and 51.4% of TB cases), and 95 of the 212 isolates (44.8%) shared an RFLP pattern with at least 1 other case and could be grouped into clusters. Risk factors associated with clustering were age <35 years (odds ratio [OR] 3.3, 95% confidence interval [CI] 95% 1.8–6.1; p<0.001), injection drug use (OR 2.1, CI 95% 1.0–4.6; p = 0.04), and previous imprisonment (OR 3.1, CI 95% 1.4–7.3; p = 0.004) (*2*). In the second period, 291 cases (77.2%) were confirmed by isolation of *M. tuberculosis*. RFLP analysis was performed on 201 isolates (69.1% of culture positive-cases and 53.3% of TB cases), and 64 of the 201 patients (31.8%) were clustered, the only statistically significant risk factor for which was birth in Spain (OR 2.2, CI 95% 1.1–4.6; p = 0.027). Contact investigation was performed for 56% and 70% during each period, respectively (p = 0.01). No statistically significant differences were found between the groups of patients whose isolates were and were not available for molecular typing in each period, except during the first period a greater proportion of pulmonary rather than extrapulmonary isolates was available for fingerprinting (84.4% vs. 71.6%, p<0.05).

Comparison between the 2 study periods showed that the decline in overall case rate was associated with a decrease in the rate of clustered cases from 7.0/100,000 to 4.4/100,000 (p<0.001) (Table 2). The nonclustered case rates did not statistically differ between the 2 periods (p = 0.45). Overall incidence of TB among the Spanish-born

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Table 1. Characteristics of tuberculosis patients during 2 periods in 3 urban districts of Madrid, Spain

Characteristic	Years		p value
	1997–1999, no. (%), n = 412	2002–2004, no. (%), n = 377	
Gender			
Male	281(68.2)	244 (64.7)	
Female	131 (31.8)	133 (35.3)	0.33
Age group, y*			
<35	178 (43.2)	159 (42.2)	
35–64	151 (36.7)	133 (35.3)	
≥65	78 (18.9)	84 (22.3)	0.55
Foreign-born			
Yes	22 (5.3)	128 (34.0)	
No	390 (94.7)	249 (66.0)	<0.001
HIV status			
Positive	106 (25.7)	46 (12.2)	<0.001
Negative or unknown	306 (74.3)	331 (87.8)	
Injection drug use			
Yes	81(19.7)	33 (8.8)	<0.001
No or unknown	331 (80.3)	344 (91.2)	
Localization of tuberculosis			
Pulmonary	292 (70.9)	275 (72.9)	0.57
Extrapulmonary or unknown	120 (29.1)	102 (27.1)	

*Age unknown for 5 patients in 1997–1999 and 1 patient in 2002–2004.

population decreased from 29.9/100,000 to 20.0/100,000 ($p<0.001$), among clustered ($p<0.001$) and nonclustered cases ($p = 0.04$). Despite no significant change in the overall case rate among the foreign-born population (40.0 vs. 58.7/100,000, $p = 0.12$), the rate of nonclustered cases increased significantly between the 2 periods ($p = 0.02$).

Conclusions

Our data show a significant decrease in the incidence of clustered TB cases from 1997–1999 to 2002–2004. Clustering in urban areas can be used as a surrogate measure for recent transmission (*3*,*4*). This reduction has had an important epidemiologic effect on the overall TB case rate in Madrid. Among Spanish-born persons, the incidence of clustered and nonclustered cases declined significantly, reflecting a significant decrease in case rate. However, among foreign-born persons, overall TB incidence increased between the 2 study periods; rate of nonclustered cases increased significantly, as did rate of clustered cases, although less dramatically. Both changes are affecting overall incidence of TB in this population.

The relative rates of recent transmission and reactivation of disease have major implications for TB control. Studies in other cities with similar overall case rates have demonstrated that decreasing the incidence of TB is possible if recent transmission is adequately controlled. After implementation of measures to control recent transmission in San Francisco, TB case rate declined from 46.0/100,000 in 1991 to 29.8/100,000 in 1997 (*5*). Similarly, in New York the number of TB cases declined by 65% from 1992 to 2000 after strict measures to identify and treat active cases were introduced (*6*). In contrast, other studies have demonstrated declining rates of disease caused predominantly by reactivation of past infections (*3*,*7*).

Another study in San Francisco (*4*) showed that the intensification of control measures decreased the overall incidence of TB by reducing number of clustered cases, which plateaued at 3 clustered cases/100,000. In our study, the rate of clustered TB cases decreased significantly between the 2 study periods, from 7.0 to 4.4 cases/100,000. We believe that directing control measures toward specific demographic subgroups remains an opportunity for reducing the rate of TB transmission in Madrid.

Overall rate of nonclustered cases did not change over the length of the study; in fact, this rate increased among foreign-born persons, most of whom were <35 years of age. Although we may be underestimating the percentage of foreign-born persons involved in recent transmission (because of the difficulty in surveying this highly mobile population), most cases in persons from countries of origin with high TB endemicity are likely to have been caused by reactivation of TB. As a consequence, greater effort is required to identify and treat latent TB infection in immigrant communities.

In conclusion, incidence of TB in Madrid decreased from 1997 through 2004, predominantly as a result of declining rates of recent transmission among the native Spanish-born population. The reduced incidence of clustered cases coincided with an increased number of TB cases among foreigners and likely indicates introduction of new strains of *M. tuberculosis* into the community. To further control TB, recent transmission of TB must be reduced by intensifying measures to identify contacts. In addition, a strategy is needed for screening for TB infection and case finding among foreign-born persons when they first con-

Table 2. Overall and clustered tuberculosis case rates during 2 periods in 3 urban districts of Madrid, Spain*

Characteristic	Case rate/100,000 persons†					
	Overall	p value	Clustered	p value	Nonclustered	p value
All cases						
Period 1	30.2	<0.001	7.0	<0.001	8.6	0.45
Period 2	25.7		4.4		9.3	
Gender						
Male						
Period 1	43.5	0.01	9.4	0.08	11.9	0.91
Period 2	34.8		6.6		12.0	
Female						
Period 1	18.4	0.70	4.8	0.02	5.6	0.26
Period 2	17.4		2.4		6.9	
Age, y						
<35						
Period 1	28.4	0.25	8.4	0.03	5.4	0.007
Period 2	24.9		5.2		9.4	
35–64						
Period 1	30.2	0.11	5.6	0.41	10.5	0.28
Period 2	24.8		4.3		8.2	
>64 y						
Period 1	32.4	0.52	4.6	0.16	12.5	0.81
Period 2	28.9		2.8		11.4	
Nationality						
Spanish-born						
Period 1	29.9	<0.001	7.2	<0.001	8.6	0.04
Period 2	20.0		3.9		6.3	
Foreign-born						
Period 1	40.0	0.12	1.8	0.23	9.1	0.02
Period 2	58.7		7.3		26.6	

*Period 1, 1997–1999; period 2, 2002–2004. All rates (overall, clustered, and nonclustered) refer to average incidence rates/100,000 persons during per the 3-year study period.
†Overall rates refer to all tuberculosis cases. Clustered and nonclustered case rates were calculated only in cases with restriction fragment length polymorphism analysis.

tact the health system in Spain. Findings such as these from similar long-term, population-based molecular epidemiologic studies can be a powerful tool for continued improvement of TB control programs.

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Influenza A Virus Infections in Land Birds, People's Republic of China

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Water birds are considered the reservoir for avian influenza viruses. We examined this assumption by sampling and real-time reverse transcription–PCR testing of 939 Asian land birds of 153 species. Influenza A infection was found, particularly among migratory species. Surveillance programs for monitoring spread of these viruses need to be redesigned.

Avian influenza virus ecology has long regarded waterbirds as a primary reservoir. Although the benchmark study detailed prevalences across all taxa (*1*), subsequent studies have focused exclusively on waterbirds (*2*) with few exceptions (*3*,*4*). We reexamined these assumptions on the basis of a broad sampling of bird diversity in Southeast Asia, where bird-borne influenza viruses are of particular concern (*5*). We sampled and tested diverse land birds for influenza A virus infection and showed that land birds also harbor infections with these viruses. Birds in these taxa are not irrelevant in virus transmission and should form an integral part of avian influenza surveillance and monitoring programs.

The Study

During 2004–2007, as part of a broader biodiversity survey and inventory program, we sampled birds from mostly forested sites in Guangxi and Guizhou Provinces in the southern part of the People's Republic of China (Figure). Sampling was conducted by mist netting and selective harvesting with shotguns; all birds in the study were apparently healthy and behaving normally at the time of collection. Because initial sampling was focused on endoparasite communities, samples from 2004–2005 consisted of complete gastrointestinal tracts frozen in liquid nitrogen. In 2006–2007, sampling was conducted specifically for viruses. Cloacal swabs were collected in 2006 and buccal–cloacal swabs were collected in 2007. All swabs were preserved in 95% ethanol.

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A total of 184 samples were collected from Jing Xi municipality in Guangxi (21.122°N, 105.964°E) in 2004, 130 from Shiwandashan Nature Reserve in Guangxi (21.840°N, 107.880°E) in 2005, 103 from Dashahe Nature Reserve in Guizhou (29.167°N, 107.575°E) in 2006, 194 from Kuan Kuoshui Nature Reserve in Guizhou (28.226°N, 107.160°E) in 2006, and 328 from Shuipu village, Guizhou Province (25.485°N, 107.882°E) in 2007 (Figure). Samples were tested for influenza A virus by real-time reverse transcription–PCR (*6*) in 2 diagnostic laboratories (Southeast Poultry Research Laboratory, US Department of Agriculture, Athens, GA, USA, and National Wildlife Health Center, US Geological Survey, Madison, WI, USA).

Of 939 samples tested, 24 were positive for influenza A viruses (prevalence 2.3%, Table; complete summary in online Appendix Table, available from www.cdc.gov/content/14/10/1544-appT.htm). If migratory behavior (species classified as migratory or nonmigratory on the basis of descriptions by MacKinnon and Phillipps [*7*]), was considered, 11 (4.8%) of 231 samples from species showing marked seasonal migrations were influenza positive. However, only 13 (1.8%) of 708 samples from nonmigratory species were positive. The cumulative binomial probability that such a high number (*11*) of positive samples would result among the 231 migratory-species samples, were the true prevalence to be 1.8%, is low ($p = 0.0013$). Thus, migratory species appear to have higher influenza infection rates. In terms of general habitat use (*7*), open-country species were slightly more prone to be influenza positive (8 [2.9%] of 274 samples) than forest species (16 [2.4%] of 665 samples), but the difference was not significant (cumulative binomial probability, $p>0.05$). Interactions between

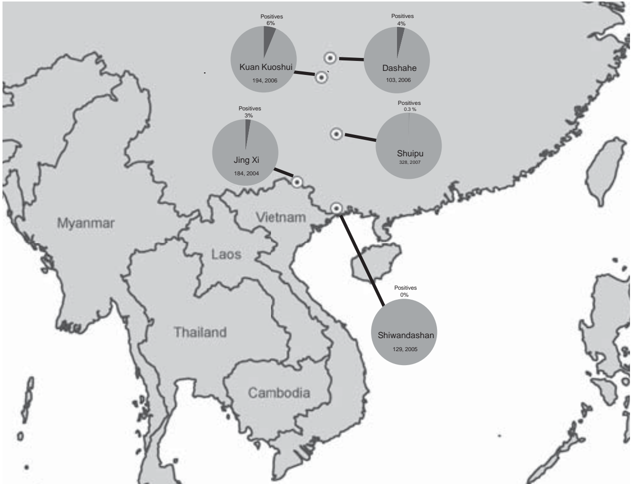

Figure. Southeastern Asia, showing 5 sites in the People's Republic of China where land birds were collected and tested for influenza A virus. Prevalence values were 4% (n = 103) in Dashahe in 2006; 6% (n = 194) in Kuan Kuoshui in 2006; 0.3% (n = 328) in Shuipu in 2007; 3% (n = 184) in Jing Xi, in 2004; and 0% (n = 130) in Shiwandashan in 2005.

Table. Prevalence of influenza A virus in avian orders and families at 5 sites, People's Republic of China

Order	Family	Location, no. positive/no. tested				
		Dashahe	Jing Xi	Kuan Kuoshui	Shiwandashan	Shuipu
Apodiformes	Apodidae	0/4				
Caprimulgiformes	Caprimulgidae				0/2	0/1
Charadriiformes	Scolopacidae		0/1			
Ciconiiformes	Ardeidae		0/1		0/1	
Columbiformes	Columbidae	0/1	0/1	0/1	0/1	
Coraciiformes	Alcedinidae		0/1		0/1	0/4
Cuculiformes	Cuculidae			0/1	0/1	
Gruiformes	Rallidae					0/1
Passeriformes	Aegithalidae	0/3				0/11
	Aegithinidae		0/1			0/2
	Campephagidae	0/1	0/4	0/1	0/8	0/4
	Cinclidae	0/5				
	Corvidae	0/2	0/1	0/3		
	Dicaeidae				0/6	
	Dicruridae		0/8		0/1	
	Emberizidae	0/10		3/18		0/20
	Estrildidae					0/13
	Fringillidae			0/1		0/6
	Hirundinidae					0/3
	Laniidae					0/1
	Monarchidae		0/6		0/8	
	Motacillidae	0/3	1/4	0/1		0/12
	Muscicapidae	2/18	2/42	1/26	0/31	1/55
	Nectariniidae			0/1	0/7	0/2
	Panuridae	0/2	0/1	0/11		0/6
	Paridae	0/2	0/1	1/20		0/10
	Passeridae	1/1		1/1		0/11
	Pycnonotidae	0/9	0/8	0/4	0/18	0/47
	Sturnidae	0/1				
	Sylviidae	1/20	1/34	2/21	0/13	1/20
	Timaliidae	0/20	1/64	3/76	0/25	1/76
	Turdidae					0/2
	Zosteropidae			1/1	0/1	0/11
Piciformes	Capitonidae				0/1	
	Picidae	0/1	0/5	0/6	0/4	0/11
Podicipediformes	Podicipedidae			0/1		
Trogoniformes	Trogonidae		0/1			

migratory behavior and habitat use were not significant (contingency test, $p>0.05$). Although all infections detected were among songbirds (Passeriformes), the sampling also concentrated on songbirds (94.3%). Thus, we could not test adequately the hypothesis that influenza prevalence was equivalent between songbirds and other birds.

An obvious question is whether the influenza A viruses we detected belong to the highly pathogenic subtype H5N1 strain currently circulating across much of Asia. All samples were negative for the H5 subtype by real-time reverse transcription–PCR (*6*), although this result does not exclude the possibility that H5 viruses were among the positive samples. The preservation status of samples we tested prevented virus isolation or full, strain-level characterization of influenza viruses.

Conclusions

The subtype H5N1 strain of influenza virus has spread rapidly and has been detected across much of central and southern Eurasia. Although movements of wild birds have been implicated in this spread (*8*), other studies question (*9*,*10*) or contradict (*11*) this idea. An important part of the argument centers on the question of the occurrence of the virus in wild birds without obvious illness, which can be difficult to interpret given the low prevalence of influenza. For instance, a recent study based on sampling >13,000 migratory birds in China detected the subtype H5N1 strain of influenza virus only 8 times (*12*), and similar results have been obtained elsewhere (*2*). Our study, although not successful in characterizing influenza viruses to specific strains, nonetheless shows that influenza A virus infection occurs in more bird species than previously assumed and that influenza A infections can be found in birds that behave normally and show no sign of illness.

Although a review of avian influenza virus ecology (*1*) discussed the occurrence of influenza viruses across all groups of birds (and other vertebrates), subsequent studies have assumed that waterbirds are the primary reservoir (*8*,*13*,*14*). In this study, a broad sample of land birds yielded frequent influenza-positive results. Although waterbirds could have higher prevalences, we have demonstrated broad occurrence of influenza viruses in diverse taxa of

Passeriformes (songbirds) in Southeast Asia. This result suggests that land birds may also be a major reservoir of influenza viruses.

We have taken a step toward a more complete understanding of influenza virus ecology among wild birds. Our partial survey of influenza virus distributions across the rich avifaunas of the southern region of China demonstrated frequent infections. This result contrasts with the current dogma in the influenza surveillance community. We suggest that to be effective future surveillance efforts will need to include the full diversity of wild birds.

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Invasive Group B Streptococcal Infections in Infants, France

Claire Poyart, Hélène Réglier-Poupet, Asmaa Tazi, Annick Billoët, Nicolas Dmytruk, Philippe Bidet, Edouard Bingen, Josette Raymond, and Patrick Trieu-Cuot

Clinical features and molecular characterization of 109 group B streptococci causing neonatal invasive infections were determined over an 18-month period in France. Sixty-four percent of the strains were from late-onset infections, and 75% were capsular type III. The hypervirulent clone ST-17 was recovered in 80% of meningitis cases.

Group B *Streptococcus* (GBS) is the leading cause of infectious illness among newborns. Invasive infections in neonates can result in pneumonia, sepsis, or meningitis. Early-onset disease (EOD) occurs within the first week. Late-onset disease (LOD) occurs after the first week and accounts for most meningitis cases and deaths (*1*). Because recommendations for intrapartum antibioprophylaxis (IAP) for mothers in labor at risk for GBS infection have been widely implemented in many countries, the incidence of EOD has declined to <1/1,000 births, but the incidence of LOD has remained unchanged (*2*). To date, 10 capsular serotypes have been described (Ia, Ib, and II–IX). Among these, serotype III is of particular importance because it is responsible for a substantial proportion of EOD and most cases of LOD (*3–8*). Different studies have suggested that most neonatal invasive diseases and almost all cases of meningitis are caused by a limited number of strains belonging to a homogeneous serotype III clone. This clone is defined by multilocus sequence typing (MLST) analyses as ST-17, the so-called highly virulent clone (*4–8*). However, data available in Europe are limited regarding the distribution of GBS genotypes among invasive isolates recovered from neonates.

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We describe clinical characteristics, capsular type, and MLST allelic and antimicrobial drug-susceptibility profiles of 109 nonredundant GBS isolates that caused neonatal invasive infections. These isolates were collected during an active surveillance performed in France from May 2006 through December 2007.

The Study

Clinical data on 109 infants up to 4 months of age were analyzed. Sepsis was defined as GBS bacteremia in the presence of consistent clinical signs and symptoms. Meningitis was diagnosed if GBS was recovered from cerebrospinal fluid. GBS isolates were identified by using a commercial Lancefield group–specific latex agglutination test. Capsular typing was performed by a multiplex PCR as described (*9*), and the hypervirulent ST-17 clone was detected by real-time PCR, as reported (*6*). Susceptibility testing, antibiograms, and MICs were performed according to Clinical and Laboratory Standards Institute recommendations (www.clsi.org). Antimicrobial drug–resistance genes were detected by using the multiplex PCR as described (*10*). Statistical analysis was performed according to the Fisher exact and χ^2 tests. A p value of <0.05 was used as the threshold for statistical significance.

We studied 109 GBS strains responsible for neonatal invasive infections; 36% (n = 39) and 64% (n = 70) were responsible for EOD and LOD, respectively (Table). Eighty percent of EOD cases occurred during the first 24 hours after birth, with a male:female ratio of 0.9; 72% were associated with sepsis, and 28% with meningitis. Maternal cultures obtained in the last 6 weeks before delivery were available for 64% of the cases. Positive GBS cultures were detected in only 11% of the cases. One death associated with meningitis occurred in an infant with EOD. A capsular serotype was assigned to all isolates with a distribution as follows: types III (61.5%) and Ia (28.2%) were predominant compared to types Ib (5.1%), II (2.5%), and V (2.5%) (Table). Capsular types IV, and VI–IX were not found. EOD meningitis GBS strains were of type III in 81.8% of isolates, and all these strains were ST-17 positive.

LOD had a male:female ratio of 1.15, and 82.6% of cases occurred during the first 8 weeks of life, with a peak (63%) at 4–8 weeks (data not shown). Sepsis occurred in 27.1% of LOD cases and meningitis in 65.7%. In 5 cases of LOD, less frequent manifestations were observed: sepsis was associated with parotitis (2 cases), osteomyelitis (1), spondylodiscitis (1), and orchitis (1). Three cases (4.5%) of recurrent invasive infections were reported. For 2 of these 3 cases, the first episode was early meningitis with a relapse of meningitis 2–3 weeks later, despite correct antimicrobial drug treatment. The third case was a late-onset sepsis that relapsed as a sepsis after the infant had received 3 weeks of amoxicillin. None of these infants was fed breast milk,

Table. Characterization of the 109 GBS strains isolated from neonatal invasive infections, France, 2006–2007*

Origin of strains (no. isolates)	CPS (no. isolates)					
	Ia (16)	Ib (7)	II (1)	III (82)	V (3)	ST-17 (75)
EOD ≤7 d (39)						
Sepsis (28)	9	2	1	15	1	13
Meningitis (11)	2	0	0	9	0	9
LOD >7 d (70)						
Sepsis (19)	2	1	0	15	1	13
Meningitis (46)	3	3	0	40	0	37
Other† (5)	0	1	0	3	1	3

*GBS, group B *Streptococcus*; CPS, capsular serotype; EOD, early-onset disease; LOD, late-onset disease.
†Sepsis was associated with 2 cases of parotitis, 1 case of osteomyelitis, 1 of spondylodiscitis, and 1 of orchitis.

which ruled out the possibility of contamination by this route. The death rate for LOD was 14.5%; 90% of deaths were associated with meningitis. Capsular type distribution of GBS LOD isolates was as follows: type III was largely predominant (83%) compared with types Ia (7.4%), Ib (4.5%), and V (1.5%) (Table). Among strains responsible for meningitis, 87% were of type III and almost all (92.5%) belonged to the hypervirulent ST-17 clone.

All 109 GBS strains tested were susceptible to penicillin (MIC_{90} 0.016 mg/L), amoxicillin (MIC_{90} 0.016 mg/L), cefotaxim (MIC_{90} 0.016 μg/mL), imipenem (MIC_{90} 0.032 μg/mL), rifampin (MIC_{90} 0.032 μg/mL), vancomycin (MIC_{90} 0.75 μg/mL), and displayed low-level resistance to gentamicin (MIC_{90} 8 μg/mL). Also, 95.5% were resistant to tetracycline because of the presence of *tet*(M) associated with *tet*(O) or *tet*(L) in 3 and 1 strains, respectively. Resistance to erythromycin was detected in 13.8% of the isolates and was not correlated with the capsular type or the onset of disease. Erythromycin resistance was caused by the presence of *mef*(A) (46.6%), *erm*(A) (26.6%), or *erm*(B) (20%).

Conclusions

In France, screening of pregnant women for GBS colonization and IAP for women detected positive was implemented in 2001 but, despite these recommendations, EOD continues to occur (*11*). In this report, 36% of cases were EOD. For 71% of EOD cases, maternal vaginal screening before delivery had not been conducted or was negative for GBS, thus likely explaining the persistence of EOD, as already suggested by others (*12*,*13*). In our study, LOD represents the majority of cases (64%), which is consistent with findings in countries where a screening approach, together with IAP, was adopted (*2*,*11*,*13*).

Clinical symptoms were significantly associated with the time of infection onset: EOD was mostly associated with sepsis (72%), whereas LOD was more frequently responsible for meningitis (65.7%) ($p<0.01$). Deaths, all associated with meningitis, were higher in LOD (14.5%) than in EOD cases (2.5%).

The predominance of capsular type III among infants with meningitis is well-known (*3*,*5*–*8*,*14*,*15*). In our study, type III accounted for 83% of LOD and was significantly associated with meningitis (85.9%; $p<0.01$) in both EOD and LOD. Moreover, the hypervirulent clone ST-17 was significantly predominant among LOD cases (75%; $p<0.03$) and accounted for 93% of GBS type III strains responsible for meningitis. This overrepresentation of ST-17 among invasive neonatal strains is now well recognized worldwide and highlights the fact that this clone is well adapted to neonate pathogenesis and may possesses specific virulence traits that enhance its invasiveness in this population (*5*–*8*,*14*,*15*). Early detection of this clone among colonizing strains in pregnant women or in neonates at delivery may therefore constitute the basis for developing new prevention strategies. An attractive alternative to IAP is vaccinating young women to subsequently protect neonates against GBS infections. Conjugate vaccines composed of capsular polysaccharides and tetanus toxoid have already been evaluated (*1*). Recent studies have suggested that protein antigens induce protective immunity in animal models, and surface proteins common to many strains would have a potential role in vaccine development (*1*). For this reason immunogenic antigens specific to the ST-17 clone should be considered in designs of future vaccine.

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Owner Valuation of Rabies Vaccination of Dogs, Chad

Salome Dürr, Martin I. Meltzer, Rolande Mindekem, and Jakob Zinsstag

We estimated the association between amount charged and probability that dog owners in N'Djaména, Chad, would have their dogs vaccinated against rabies. Owners would pay ≈400–700 CFA francs (US $0.78–$1.36)/animal. To vaccinate ≥70% of dogs, and thus interrupt rabies transmission, health officials should substantially subsidize these vaccinations.

Canine rabies globally causes an estimated 55,000 human deaths each year; 23,750 (≈43%) of which occur in Africa (*1*). To eliminate rabies virus in dog populations, and thus reduce the risk for human rabies, the World Health Organization (WHO) recommends dog rabies vaccination coverage of 70% (*2*). However, in most sub-Saharan countries, per capita expenditures on human health care are typically <$50/year (*3*), which makes securing funding to achieve the WHO target for dog vaccination challenging.

One way to fund dog rabies vaccination programs is to charge owners a fee for each dog vaccinated. However, the higher the fee, the lower the compliance is likely to be. To estimate the association between the amount charged to dog owners and the probability of vaccination (i.e., vaccination coverage), we collected data from 3 observational studies (2001, 2002, 2006) and 1 survey of dog owners (2006) (*4,7*; Table). We then estimated the maximum amount that could be charged to owners (cost recovery) and still achieve a minimum of 70% of dogs vaccinated.

The Study

We used data collected in the capital of Chad, N'Djaména, which in 2001 had a human population of ≈776,000 and a dog population of ≈23,600 (*4*). Dog rabies is endemic to Chad; before the vaccination campaigns, the prevalence of dog rabies was ≈1.4–1.7 cases/1,000 unvaccinated dogs (*5,6*).

We obtained direct observations of the association between compliance (i.e., percentage of dogs vaccinated) and amount charged to owners from 2 pilot dog vaccination campaigns held in N'Djaména in 2002 and 2006 (*7*; unpub. data). Both campaigns followed similar protocols. Each campaign covered the same 3 city quarters, which had high-density dog populations (*4*). Only owned animals were vaccinated, but owned dogs comprise 90%–99% of all dogs in N'Djaména (*7*). Owners brought their animals (dogs, cats, monkeys) to 1 of 10 vaccination sites. In the 2002 campaign, vaccinations were free to owners (*7*); in the 2006 campaign, owners were charged 2,000 CFA francs (US $3.88)/animal vaccinated (unpub. data). (Exchange rate US $1 = 515.71 CFA francs as of February 2007; www.oanda.com/convert/classic) For each campaign, the percentage of dogs vaccinated was estimated by using a capture–recap-

Table. Characteristics of 356 persons interviewed and their households, N'Djaména, Chad, 2006

Characteristic	No. (%)
No. persons/household	
1–10	230 (65)
11–20	107 (30)
21–30	10 (3)
30–35	1 (0)
Unknown	8 (2)
Gender of persons interviewed*	
Female	165 (46)
Male	190 (53)
Unknown	1 (1)
No. animals/household	
Dogs	
0	7 (2)
1	278 (78)
2	65 (18)
3	6 (2)
Cats	
0	341 (96)
1	14 (4)
2	1 (0)
Monkeys	
0	346 (97)
1	10 (3)
Age of animals, y	
<1	101 (22)
1–<3	155 (34)
3–<6	134 (30)
≥6	48 (11)
Unknown	12 (3)
Average	3.37
Sex of animals	
Male	346 (77)
Female	101 (22)
Unknown	3 (1)
Animals vaccinated ≥1 time	
Yes	314 (70)
Within past year†	197 (44)
Vaccinated during a campaign	121 (27)
No	132 (29)
Unknown	4 (1)

*Mean age of persons interviewed 33.7 y (range 13–80 y).
†Confirmed by inspection of certificate of vaccination.

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ture method (*7*; unpub. data). For the 2002 free-to-owners campaign, 71%–87% (95% confidence interval [CI] 64%–89%; mean 79%) of all dogs (owned and unowned) were vaccinated in 2 of the zones (1 zone per quarter) included in the campaign (*7*). For the 2006 campaign, in which owners were charged, the mean vaccination coverage among all dogs was estimated at 24% (95% CI 0.13%–24.82%) (unpub. data). Vaccination rates for owned dogs averaged only 78% and 25% in the 2002 and 2006 campaigns, respectively (*7*; unpub. data). For this study, we used the latter estimates because we were interested in measuring owner compliance to charges for dog vaccination.

Additional observational data were obtained from a household survey conducted in 2001 (*4*), which recorded that 19% of owned dogs were vaccinated against rabies. Such vaccinations would have been given at private clinics (i.e., without a campaign). The charge for such vaccinations at the urban government-run veterinary clinic and the 3 private veterinary practices of N'Djaména was 3,000–5,000 CFA francs (US $5.82–$9.69). We used the midpoint of such charges (i.e., 4,000 CFA francs). We did not inflate the 2001 charges because we encountered problems identifying an appropriate conversion factor that considered veterinary medical services.

During the 2006 campaign we surveyed dog owners by using a short questionnaire (online Technical Appendix, available from www.cdc.gov/EID/content/13/10/1650-Techapp.pdf). The survey was conducted in the vaccination zones; households (containing at least 1 animal) were chosen randomly. The questions (written in French) were translated, when needed, into local languages by 4 interviewers. One question asked owners how much they were willing to pay for the vaccination of their animals.

We graphed the 3 observational data points (assuming a straight-line interpolation between points) and the reverse cumulative probability of the owner-stated amounts that they would be willing to pay for their animal to be vaccinated against rabies (Figure). An initial statistical (regression) analysis of the relationship between the amounts that owners said they would pay and the variables collected during the survey (Table) provided an adjusted r^2 value of 0.07 (data not shown). We did not perform additional statistical analyses.

Interviewed households provided 356 questionnaires from which we estimated owner-stated willingness-to-pay for pet vaccination and calculated the resultant reverse cumulative probability of having their animal vaccinated. When asked how much they would be willing to pay, 5 (1%) owners stated that they were against vaccination. We interpreted that response to indicate that such owners would, essentially, have to be paid to have their animals vaccinated.

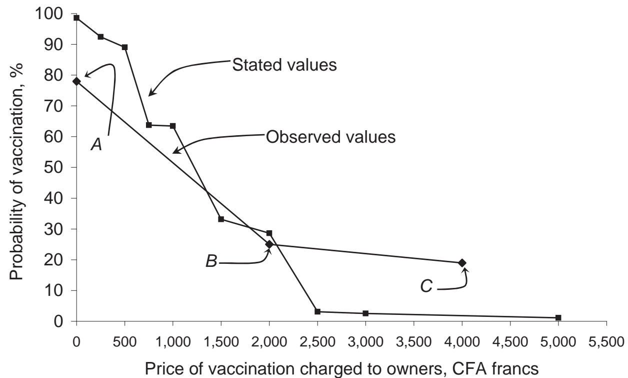

Figure. Average probability of having a dog vaccinated against rabies by charge for vaccination: observed versus owner-stated values for vaccination. The observed values of charges to vaccinate an owned dog against rabies and probability of vaccination came from 3 sources. Points A and B (recording vaccination coverage for all owned dogs vs. costs charged) come from 2 vaccination campaigns held in N'Djaména in 2002 and 2006, respectively. Point C represents the midpoint of the range of recorded 2001 clinic charges in N'Djaména for vaccinating a dog against rabies (costs not adjusted for any potential inflation). The owner-stated amounts that they would be willing to pay for their dogs to be vaccinated against rabies came from a survey of 356 households, conducted in 2006. The graph shows the reverse cumulative probability of the stated values.

When the proposed cost of vaccination was ≤1,500 CFA francs/animal vaccinated, owners were more likely to state that they would pay to have their pet vaccinated than they were to actually do it. The stated values and observed values were closest at 2,000 CFA francs (≈25% probability of animal being vaccinated) (Figure). This finding was probably because the questionnaire was administered immediately after the campaign in which owners were charged 2,000 CFA francs/animal vaccinated. For >2,000 CFA francs, the observed values indicated that compliance would be greater than that stated by owners' responses to the willingness-to-pay question. The Figure shows that to achieve a minimum of 70% of owned animals vaccinated, the maximum amount that could be charged would be ≈400 CFA francs (US $0.78) (observed values) to ≈700 CFA francs (US $1.36) (owner-stated values). Because the data shown in the Figure reflect owned animals only, to get vaccination coverage up to 70% of all animals (owned and stray), vaccination rates among owned animals would have to be >70%. To attain these higher rates, charges would have to be even lower than 400–700 CFA francs.

Conclusions

Few studies have compared what members of the general public state they are willing to pay for a public health intervention with their actual observed behavior (*8*). Direct

comparison between stated and observed behavior, as influenced by charges to owner, provides public health officials with an understanding of the reliability of owner surveys.

Our study and the data used have several limitations. First, the sample sizes were quite small (*6*,*7*). Furthermore, to maintain dog vaccination rates at the WHO-recommended rate of 70%, dog vaccination campaigns would have to be held every 1–6 years, which could reduce compliance. The survey was, by design, short, but a longer questionnaire may have allowed us to better identify why 75% of respondents did not wish to pay >500 CFA francs (US $0.97).

To achieve the WHO-recommended goal, public health officials cannot charge owners more than the equivalent of 400 CFA francs (US $0.78). Full-cost recovery concepts will not ensure that enough dogs are vaccinated in Chad (or, most likely, other African countries) to interrupt rabies transmission in dogs in urban areas. Clearly, to have ≥70% of all dogs vaccinated, public health officials and policy makers must consider methods to substantially subsidize dog rabies vaccinations.

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Unexplained Deaths and Critical Illnesses of Suspected Infectious Cause, Taiwan, 2000–2005

Tsung-Hsi Wang, Kuo-Chen Wei, Donald Dah-Shyong Jiang, Chan-Hsian Chiu, Shan-Chwen Chang, and Jung-Der Wang

We report 5 years' surveillance data from the Taiwan Centers for Disease Control on unexplained deaths and critical illnesses suspected of being caused by infection. A total of 130 cases were reported; the incidence rate was 0.12 per 100,000 person-years; and infectious causes were identified for 81 cases (62%).

In 2003, the outbreak of severe acute respiratory syndrome (SARS) demonstrated that the world has become a global village in which human risk for exposure to different kinds of biological hazards is increased through frequent travel and commercial activities (*1–5*). Historically, emerging diseases occur abruptly in outbreaks of unknown cause. Although various efforts have been proposed and conducted to analyze secondary data periodically (*6–9*), they generally provide information for the less urgent decision making in health policy and may not be in time for infectious disease control. Thus, a task force is needed to provide timely and accurate diagnosis for early control of any potential epidemic infection, especially in a newly developed country like Taiwan, where the healthcare resources may not be evenly distributed and autopsy for diagnosis is not widely accepted culturally.

In 2000, the Taiwan Centers for Disease Control collaborated with academic institutions, medical examiners, local health authorities, and experts from different fields to establish a nationwide surveillance center for outbreak and unexplained death investigation due to unknown infectious causes (COUNEX) (Figure). This effort was to build

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Local health authorities, hospitals, or clinics report suspected infection-related cases through the "notifiable infectious disease surveillance system," "syndromic surveillance system," and toll-free notification hotline

↓

Local COUNEX committee (at 4 TCDC branches): screening and information collection

↓

Investigation task force* supported by central and contract laboratories†: investigations and data aggregation

↓

Review of medical records and all other relevant information (within 24 h) for a decision on whether an autopsy is indicated

↓

Approved by patient's family

No → Final review

Yes → Autopsy performed by designated pathologists at contract pathology laboratory at medical centers‡

No / Yes → Final review

Final review of etiologic evidence and decision by COUNEX committee at TCDC (composed of members from all 4 local branches)§

Figure. Flow of information and decision making for reported cases of unexplained death or critical illness. *If unexplained infectious causes were suspected, COUNEX mobilized an investigation team including experts, field epidemiology training program members, public health workers from the local branch of Taiwan Centers for Disease Control (TCDC), and public health authorities to proceed with further field investigation. TCDC was in charge of the investigation. †Cases were categorized into ≥1 of the following clinical syndromes: acute neurologic (encephalitis, meningitis), acute respiratory (pneumonia), acute hemorrhagic, acute diarrhea, acute jaundice (hepatitis), acute heart (myocarditis, pericarditis, endocarditis), and acute kidney-related. For every reported case, COUNEX investigators usually selected diagnostic tests relevant to a particular syndrome (www.cdc.gov.tw). Additional tests were prescribed if needed. The hospital laboratories were requested to save all remaining clinical specimens, including biopsy specimens, obtained from clinical management and send them to our reference laboratories, if indicated. ‡If an autopsy was performed, whenever possible tissue specimens were examined by pathologists of TCDC-designated medical centers and the Forensic Department of the Ministry of Justice to ensure the accuracy of the final diagnosis. Specimens were also sent for microbiologic cultures and tests as well as toxicologic examination for trace toxic chemicals, if needed. §All laboratory results and clinical, epidemiologic, and pathologic data were sent to the expert committee to determine if the etiologic agent could fully or most likely explain the disease. Otherwise, cases were categorized as unexplained. In general, histopathogic examination was the major evidence for determining cause. If case-patients could not be autopsied within 36 hours of death, laboratory results would be the most useful information for identification of cause of death.

Taiwan's capacity for detecting and responding to uncommon and unrecognized pathogens, which was conceptually the same as that of the study of Hajjeh et al. (*10*). We defined the surveillance case-patient as a previously healthy resident who died or was admitted to a hospital with a life-threatening illness possibly caused by infection of unidentified etiology. Usually the death occurred within 3 days of the patient's admission. Patients were excluded if the cause of death was noninfectious. A life-threatening illness was defined as any illness requiring admission to an intensive care unit or reported as being critical. An infectious disease is generally suspected if the case-patient has ≥1 of the characteristics such as fever, leukocytosis, histopathologic evidence of an acute infectious process or more specific symptom patterns, or infection precipitating adult respiratory distress syndrome, renal failure, or sepsis.

A total of 130 cases were reported during 2000–2005, for an annual average rate of 0.12 cases per 100,000 persons. The annual incidence rates varied by year and among 4 branches of Taiwan Centers for Disease Control (Table). The highest rate was in the eastern branch, where surveillance was conducted in a well-defined population of ≈596,119 persons. Ninety-five (73%) of the case-patients died. For 47 (49%) of those who died, an autopsy was performed, a rate much higher than the national autopsy rate of <11% (*12*). The mean age of case-patients was 33.8 years. The incidence rates varied by age group; it was highest in those 85–89 years of age, followed by those <1–4 years, and then 65–69 years, with 0.48, 0.30, and 0.23 per 100,000 person-years, respectively. Men had a higher incidence rate than women (0.16 vs. 0.10 per 100,000 person-years).

Approximately 10% of 130 case-patients and 16% of 81 patients with cases of infection had a history of animal contact; 9% of 130 case-patients and 10% of infection case-patients had a history of travel outside Taiwan within the previous 3 months. The most common initial syndromes were acute respiratory (59%), acute neurologic (22%), and acute diarrhea-related syndrome (13%). Initially, 8 patients had acute heart-related syndrome, and 11 had acute kidney-related syndrome; both of these syndromes had a 100% case-fatality rate.

The online Appendix Table (www.cdc.gov/EID/content/14/10/1653-appT.htm) lists all the infectious pathogens and noninfectious causes identified among 95 fatal cases. One third were related to bacterial infection and one fourth to viral infection; 22 remained unclassified. The proportion of explained cases was lower among patients who survived (74%) than that among patients who died (77%). The proportion of explained cases was also higher for patients who underwent autopsy (83%) than for non-autopsied patients (71%) but not statistically significantly so. Explained cases were similar to unexplained cases in terms of patient age and interval between dates of disease onset and report (median 7.2 and 6.8 days, for explained and unexplained cases, respectively). Although the overall case-fatality rate was 73%, patients were more likely to die if they had multiple organ system involvement.

We have established the infrastructure needed to detect critical and fatal cases of unknown causes; such a surveillance system is essential to identify early potential infectious threats in a period of globalization and increasing travel between countries. The contributions of our surveillance system are demonstrated by early detection and control of at least 3 outbreaks of serious viral diseases: hantavirus pulmonary syndrome, rabies, and SARS.

In 2001, a family cluster occurred in Huanlian city; dyspnea, cough, leukopenia, and pulmonary edema developed in both parents, who died. Their 16-year-old daughter was also ill, but she survived. COUNEX quickly intervened, and hantavirus pulmonary syndrome was confirmed by positive serologic test results, which led to an early control of local rodents and spread of the disease.

Taiwan has been free of human and animal rabies since 1961. However in 2002, a 45-year-old woman from mainland People's Republic of China was admitted to a hospital because of difficulty in swallowing, fear of wind (aerophobia), and numbness of the arms. Her condition was reported to the surveillance system as suspected rabies. Our

Table. Incidence rate of case-patients detected by surveillance and proportions of deaths, possible infectious causes, and autopsy, Taiwan, August 2000–March 2005

Category	Total	TCDC branch*			
		Northern	Middle	Southern	Eastern
Incidence/100,000 person-years†	0.12	0.09	0.16	0.09	0.64
Proportion of deaths among all case-patients, %	73	75	64	79	83
Proportion of infectious causes identified, %	65	63	68	68	39
Viral agents among infection cases, %	42	56	30	37	57
Bacterial agents among infection cases, %	46	36	57	47	36
Rickettsia spp. among infection cases, %	4	4	3	0	14
Proportion of causes remaining unknown, %	23	25	16	29	25
Autopsy rate among patients who died, %	49	53	43	41	67

*TCDC, Taiwan Centers for Disease Control.
†Denominators for the population under surveillance, obtained from the 2002 intercensus (*11*) and approximately the midpoint of this study period, included all people in the age groups under surveillance at the various sites and were used to calculate the incidence rate.

personnel quickly confirmed the diagnosis by reverse transcription–PCR and DNA sequence analysis of the samples from cerebrospinal fluid, saliva, and trachea while the patient was still alive (*13*). The patient had been bitten by a domesticated dog in mainland China 2 months earlier.

During the SARS outbreak in 2003, the surveillance system received reports of 6 cases; autopsies were performed on 3 patients. As a result, the correlation between clinical course and pulmonary pathology at different stages of the disease was possible, and corroborative evidence for control measures was provided (*14*).

Had the surveillance system for unexplained death and critical illness not functioned normally during these 3 outbreaks, more people in Taiwan would have been ill and died from the diseases because of the high population density on this island. This system was particularly useful for infection control at remote regions with limited resources. Most physicians in the rural eastern part of the country have less access to consultation and referral to other specialties in medical centers and teaching hospitals. Thus, they rely more on this kind of surveillance system for early detection of potential infectious threats. This was especially important for acute unexpected deaths, as was demonstrated by a higher incidence and autopsy rates in eastern Taiwan.

Throughout this project, we have increased the autopsy rate and established a population-based bank of specimens for future research. This collection could provide a better opportunity for corroboration or refutation of any previous diagnosis of infectious disease. This improved decision making in regard to control of infections was demonstrated in November 2003, when influenza virus (H5N1) was diagnosed in a patient who had a previous misdiagnosis of SARS (*15*).

Because emerging and reemerging infectious diseases may quickly travel between different countries, the system is becoming more crucial for early detection and control of potential health hazards. The system depends on close cooperation among different disciplines and staff from different agencies. Thus, education, empowerment, and good feedback incentives should be continually offered to keep this system sustainable.

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Murine Typhus and Febrile Illness, Nepal

Mark D. Zimmerman, David R. Murdoch, Patrick J. Rozmajzl, Buddha Basnyat, Christopher W. Woods, Allen L. Richards, Ram Hari Belbase, David A. Hammer, Trevor P. Anderson, and L. Barth Reller

Murine typhus was diagnosed by PCR in 50 (7%) of 756 adults with febrile illness seeking treatment at Patan Hospital in Kathmandu, Nepal. Of patients with murine typhus, 64% were women, 86% were residents of Kathmandu, and 90% were unwell during the winter. No characteristics clearly distinguished typhus patients from those with blood culture–positive enteric fever.

In 2001, we found *Salmonella enterica* serotype Typhi and *S. enterica* serotype Paratyphi A to be the most common causes of bloodstream infections among adults with febrile illness who sought treatment at Patan Hospital in Kathmandu, Nepal (*1*). Another important finding was the relatively high percentage of patients (11%) who had immunoglobulin (Ig) M antibodies against *Rickettsia typhi* in peripheral blood. Because most testing was performed on unpaired acute-phase sera, and a high percentage of seropositive results were found in a group of healthy study participants, we were uncertain whether these participants had acute murine typhus or more distant past infection.

Recent studies have shown the value of PCR for diagnosing scrub typhus (*2–4*), and a real-time assay for *R. typhi* has recently been validated (*5*). In our study, we tested archived blood samples from our febrile adult cohort (*1*) with this *R. typhi* PCR to better characterize the incidence of murine typhus and to determine whether clinical features could help distinguish murine typhus from enteric (typhoid) fever.

The Study

We studied consecutive adult patients with fever (axillary temperature >38°C; >13 years of age) who sought treatment at Patan Hospital from January 15 through March 15, 2001 (winter) and July 2 through August 10, 2001 (summer), as detailed elsewhere (*1*). The study was approved by the Nepal Health Research Council and the Institutional Review Board of the Centers for Disease Control and Prevention.

Blood from each patient was injected into blood culture bottles and serum samples were tested for *R. typhi* IgM antibodies (INDX Multi-Test Dip-S-Ticks SDLST; Integrated Diagnostics, Inc., Baltimore, MD, USA). In addition, whole blood samples (stored at −80ºC) were tested by real-time PCR for *R. typhi* at the Naval Medical Research Center, Silver Spring, MD (NMRC) and at Canterbury Health Laboratories, Christchurch, New Zealand (CHL). Details of the assay have been described elsewhere (*5*). We extracted DNA from 200 µL of whole blood and used primers and probes specific to a portion of the outer membrane protein B (*ompB*) unique to *R. typhi* to amplify and detect the target sequence in a SmartCycler (Cepheid, Sunnyvale, CA, USA) at NMRC and in a LightCycler (Roche Diagnostics, Mannheim, Germany) thermocycler at CHL. Thermocycling parameters included an initial denaturation step (2 min at 9°C) followed by 45 cycles of denaturation (94°C for 5 s) and annealing/elongation (60°C for 30 s) steps. Positive samples were defined as those that demonstrated fluorescence above background levels. Template-free controls assayed at the same time and under the same conditions as the experimental and positive control samples consistently showed negative results.

In our study, a diagnosis of murine typhus required a positive *R. typhi* PCR result; a diagnosis of enteric fever required a positive blood culture for *S.* Typhi or *S.* Paratyphi. Data from patients with murine typhus were compared with those from patients with enteric fever by using the χ^2 test or Fisher exact test for dichotomous and ordinal variables, and 2-sided Wilcoxon rank sum test and the Student *t* test for continuous variables. We used multivariable logistic regression analysis to further evaluate variables associated with murine typhus. Murine typhus was the outcome variable in the final model; other variables were those associated with the outcome with p<0.1 on bivariable analysis. Data were analyzed by using STATA version 8.2 (StataCorp, College Station, TX, USA).

We enrolled 876 patients, 370 in winter and 506 in summer. In 323 (37%) patients, a putative diagnosis was established; 117 (13%) patients had positive blood cultures for *S.* Typhi or *S.* Paratyphi A.

Whole blood samples were available for testing from 756 (86%) patients. Of these patients, 85 (11%) had *R. typhi* IgM antibodies detected in acute-phase serum samples and 50 (7%) had positive *R. typhi* PCR results; 11

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(13%) of the *R. typhi*–seropositive patients were also PCR positive. Sequencing of amplicons from 5 PCR-positive patients showed 100% similarity with the reference strain of *R. typhi* (GenBank accession no. AE017197). None of the patients with positive PCR results for *R. typhi* had a positive blood culture.

The features of the 50 patients with murine typhus and the 94 patients with enteric fever are presented in the Table; all had negative *R. typhi* PCR results. Sixteen of the murine typhus patients had chest radiographs; 6 (38%) of these patients were reported as having lung infiltrates. Of the 50 patients with murine typhus, 45 were managed as outpatients and 5 were ill enough to be admitted to a hospital. No deaths occurred.

After logistic regression analysis, only 3 variables were significantly associated with murine typhus compared

Table. Demographic, clinical, and laboratory features of patients with murine typhus and enteric fever, Nepal*

Variable	Murine typhus, n = 50	Enteric fever, n = 94	p value
Demographics			
Age, y, median (range)	28 (15–85)	22 (14–72)	0.0001
Male gender	18 (36)	61 (65)	0.001
Occupation			0.001
Housewife	17 (41)	10 (12)	
Student	7 (17)	36 (42)	
Business person	2 (5)	7 (8)	
Government employee	2 (5)	8 (9)	
Other	13 (32)	24 (28)	
Residence			<0.001
Kathmandu	31 (86)	27 (32)	
Patan	0	28 (33)	
Kathmandu Valley	4 (11)	10 (12)	
Other	1 (3)	20 (24)	
Winter season	45 (90)	27 (29)	<0.001
Admission diagnosis			<0.001
Enteric fever	21 (42)	66 (70)	
Lower respiratory tract infection	13 (26)	3 (3)	
Urinary tract infection	6 (12)	3 (3)	
Upper respiratory tract infection	3 (6)	1 (1)	
Other	7 (14)	21 (22)	
Symptoms			
Cough	33 (66)	30 (33)	<0.001
Shortness of breath	16 (32)	6 (7)	<0.001
Nausea	14 (28)	34 (37)	0.26
Diarrhea	5 (10)	16 (18)	0.24
Abdominal pain	16 (33)	25 (28)	0.58
Headache	41 (82)	78 (86)	0.56
Joint pain	12 (24)	11 (12)	0.07
Duration of symptoms, d, median (range)	5 (1–10)	5 (1–30)	0.23
Examination findings			
Temperature, °C, mean (SD)	38.9 (0.7)	38.8 (0.6)	0.54
Respiratory rate, breaths/min, mean (SD)	26 (9)	21 (5)	0.0002
Heart rate, beats/min, mean (SD)	112 (17)	105 (15)	0.02
Systolic blood pressure, mm Hg, mean (SD)	109 (17)	107 (11)	0.52
Diastolic blood pressure, mm Hg, mean (SD)	70 (10)	70 (8)	0.88
Crackles	13 (26)	8 (9)	0.006
Hepatomegaly	2 (4)	9 (10)	0.22
Splenomegaly	2 (4)	12 (13)	0.09
Rash	0	1 (1)	0.46
Laboratory findings			
Hematocrit, %, mean (SD)	39 (6)	39 (5)	0.70
Leukocyte count, cells × 10^9/L, median (IQR)	8.9 (6.2–11.1)	5.8 (4.8–7.6)	<0.001
Neutrophils, %, median (IQR)	83 (74–87)	68 (60–73)	<0.001
Lymphocytes, %, median (IQR)	13 (9–22)	27 (20–35)	<0.001
Monocytes, %, median (IQR)	2.5 (1–5)	2 (0–4)	0.57
Eosinophils, %, median (IQR)	0.5 (0–2)	0 (0–2)	0.80

*Data are no. (%) unless otherwise stated. SD, standard deviation; IQR, interquartile range.

with enteric fever. These variables were age (for each increase by 1 year) (odds ratio [OR] 1.07, 95% confidence interval [CI] 1.00–1.16, p = 0.05); Kathmandu residence (OR 14.37, 95% CI 1.07–193.39, p = 0.05); and winter season (OR 28.93, 95% CI 2.47–338.93, p = 0.007).

Conclusions

We detected *R. typhi* DNA in blood from 7% of the febrile adult study population from urban Nepal. This finding is likely to be an underestimate of the actual extent of rickettsial disease because of the small volume of blood tested in each PCR and possible sample deterioration during transport and storage (between sample collection and testing). Although PCR has yet to be extensively evaluated for the diagnosis of murine typhus (*5–8*), a sizeable body of evidence supports the high sensitivity and specificity of PCR for the diagnosis of rickettsial diseases (*2,4,9,10*). The real-time PCR used in our study has a high analytical sensitivity and specificity for *R. typhi* (*5*), and sequencing of amplicons from our patients further supports the specificity of the assay. In addition, none of our patients with murine typhus had positive blood cultures.

The results of this and other (*11,12*) studies indicate that murine typhus is an important endemic infection in Nepal. Although our study did not extend throughout the full year, murine typhus was more common in winter than in summer. This finding contrasts with the summer–autumn predominance reported in other regions (*13,14*). We also noted a clear predominance of cases from Kathmandu and none from the Patan side of the city, despite the fact that the latter is the main catchment area for Patan Hospital. It is possible that an outbreak of murine typhus occurred in Kathmandu during the winter of 2001, and epidemiologic studies are needed to clarify whether there is a focus of murine typhus activity in Kathmandu. In Nepal, enteric fever is one of the most common causes of febrile illness (*1,15*). We identified no reliable clinical marker to distinguish murine typhus from enteric fever, and the classic clinical triad of rickettsial diseases (headache, fever, and rash) was not detected in any of our patients. Murine typhus should be considered as an alternative diagnosis in patients with suspected enteric fever in Nepal. This diagnosis is especially important given that first-line antimicrobial drug therapy is different for the 2 diseases.

Our study highlights the importance of rickettsial infections as a cause of febrile illness in Kathmandu. Further epidemiologic and ecologic studies are needed to better clarify the features of murine typhus in Nepal.

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Dr Zimmerman is executive director of the Nick Simons Institute, an organization working in Nepal with a mission to train and support skilled, compassionate, rural healthcare workers. He was previously the medical director of Patan Hospital, Kathmandu, Nepal.

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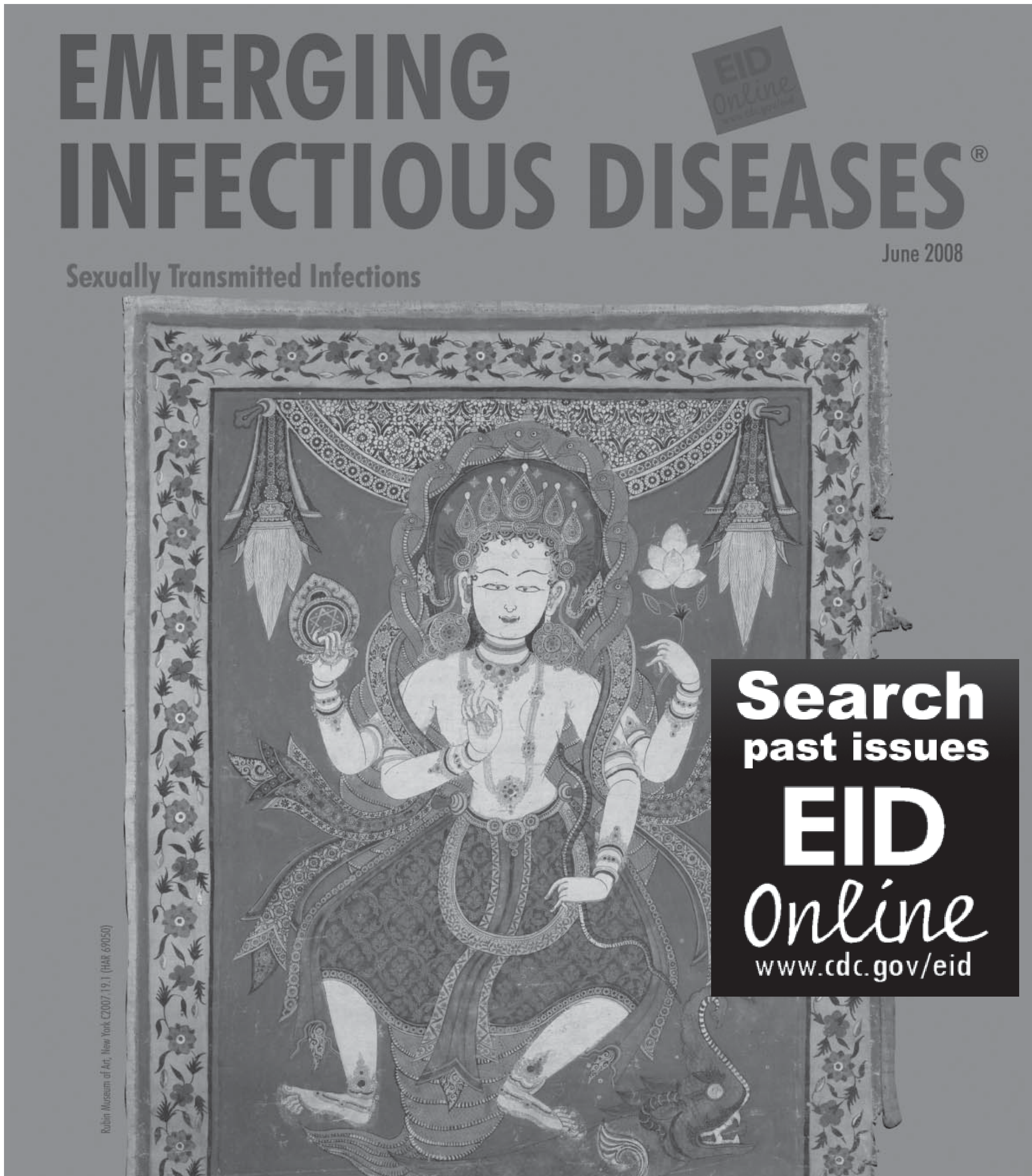

Effects of School Closures, 2008 Winter Influenza Season, Hong Kong

Benjamin J. Cowling, Eric H.Y. Lau, Conrad L.H. Lam, Calvin K.Y. Cheng, Jana Kovar, Kwok Hung Chan, J.S. Malik Peiris, and Gabriel M. Leung

In winter 2008, kindergartens and primary schools in Hong Kong were closed for 2 weeks after media coverage indicated that 3 children had died, apparently from influenza. We examined prospective influenza surveillance data before, during, and after the closure. We did not find a substantial effect on community transmission.

Hong Kong, Special Administrative Region, People's Republic of China, is a subtropical city in Southeast Asia at the epicenter of global influenza activity (*1*). Epidemiologically, influenza usually displays biannual seasonality, consisting of a winter peak typically between January and March and a summer peak in June and July, often with an elevated plateau in April and May between the winter and summer peaks (*2–4*).

On the evening of March 12, 2008, after 3 children had died, apparently from influenza, the government of Hong Kong announced that all primary schools, special schools, kindergartens, and day nurseries would close the following day for a total of 2 weeks, 1 week earlier than the scheduled start of the annual week-long Easter break (*5*,*6*).

The Study

We reviewed prospective surveillance data on influenza and influenza-like illness activity during the 2008 winter influenza season. We then considered the effects of the school closures on community transmission.

As elsewhere in the Northern Hemisphere, the 2007–08 strains of influenza virus circulating in Hong Kong were closely related to A/H1N1/Brisbane/59/2007, A/H1N1/Soloman Islands/3/2006-like, A/H3N2/Brisbane/10/2007, B/Yamagata/16/88-like, and B/Malaysia/2506/2004-like. These strains were not well matched to the trivalent inactivated vaccine specified for the season.

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Surveillance data from different settings before, during, and after the period of school closures are shown in the Figure. Laboratory isolation of influenza viruses in children (panel A) and adults (panel B) show that the influenza season began in January, rose to a peak in late February, and was already waning by the time the decision was made to close schools, as temperatures and relative humidity were increasing (*8*). Influenza circulation has remained at a low baseline level since schools reopened in early April. Absenteeism rates in sentinel childcare centers and primary schools gradually rose to maximums of 7.9% and 3.5%, respectively, before the school closures and returned to low levels after the closures (data not shown). Similarly, influenza consultation rates at public and private outpatient clinics (panel C) peaked before the closures and generally reflected the reference laboratory data, except for a dip during Chinese New Year, when many sentinel practices were closed.

When compared with the influenza seasons of the preceding 9 years, the 2008 winter influenza season was moderately severe in terms of outpatient consultations (online Appendix Figure, available from www.cdc.gov/EID/content/14/10/1660-appF.htm). Influenza hospital admission rates in children ≤4 years reached peak levels of 30/100,000 population in 2006 and 41/100,000 in 2007, both mild seasons. These rates were similar to the peak level of 39/100,000 in 2008 (Figure, panel D) (*6*). The elderly appeared to have been less affected, with no clear rate increases noted by febrile sentinel surveillance in elderly care homes and generally low influenza-related admission rates in this age group (data not shown).

Panel E of the Figure shows daily estimates of the effective reproductive number, or R_t, based on a simple method (*9*) that we applied to daily interpolations of the laboratory and outpatient sentinel data. We used a Weibull model for the serial interval with mean of 3.6 days and standard deviation of 1.6 days, based on data from a recent community study (*10*). The effective reproductive number on day *t* can be interpreted as the average number of new persons infected by an infector who had symptom onset on day *t*. Therefore, a reproductive number >1 implies that an epidemic will grow in the short term, whereas a number <1 implies that an epidemic will die out. These trends, in particular the lack of any apparent negative inflection point during the entire 2-week period of school closure, suggest that the effect of the intervention was not substantial. Trends in estimated R_t were similar if serial intervals of mean 2.5 or 2.0 days were assumed.

Conclusions

Although we can only speculate, given the limitations of an uncontrolled natural experiment on the population level, routine surveillance data did not detect a large effect

from the school closures. In particular, we noted a decline in laboratory isolations of influenza viruses that preceded the intervention and the lack of association between school closures and R_t. In fact, sentinel data may not accurately represent the incidence of influenza in the underlying population because, for example, other cocirculating upper respiratory viruses contribute to overall influenza-like illness consultation rates. Laboratory data, however, should be less affected, and extra testing in response to the heightened awareness of influenza activity might have artifactually lowered the positivity rate. The epidemic curves generated from the surveillance data showed a decline in cases that may have naturally concluded without any intervention. We note the difficulty of making inferences directly from changes in epidemic curves because changes in the epidemic curve may lag behind changes in the underlying transmission dynamics by at least 1 serial interval, as has previously been shown for severe acute respiratory syndrome (*9*,*11*). Although the estimates of R_t (panel E) are crude, the estimated values of 1.2–1.5 during the rising phase of the 2008 winter epidemic in Hong Kong are slightly lower than previous estimates for interpandemic influenza (*12*,*13*), perhaps because of the low time-dependent resolution of the weekly aggregation of surveillance data.

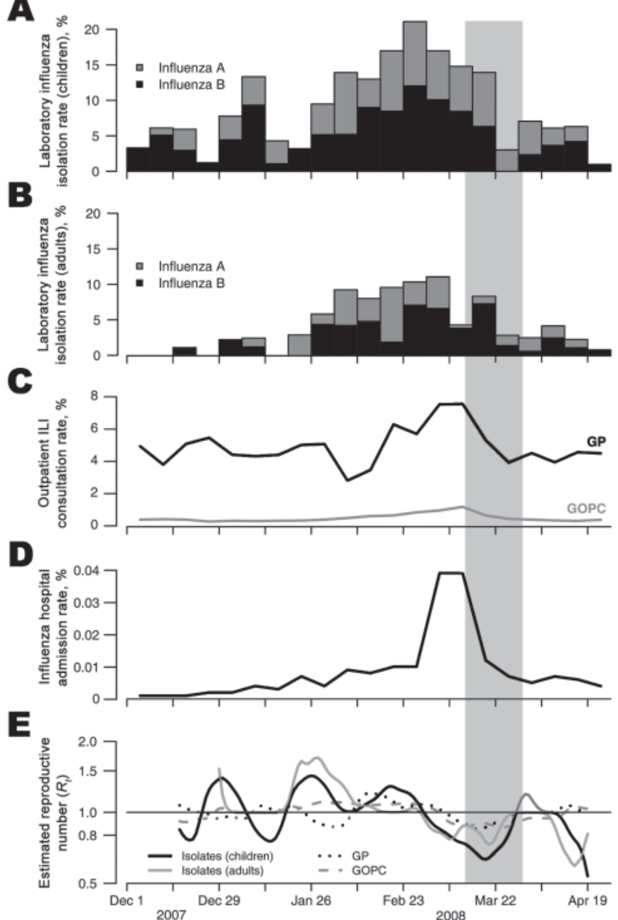

Figure. Influenza surveillance data from December 1, 2007, through April 26, 2008, including the 2-week school closure period (gray vertical bar): A) Proportion of influenza A and B isolations (by date of collection) among all children's specimens that were submitted to the World Health Organization (WHO) reference laboratory at Queen Mary Hospital (most specimens are referred from local hospitals). B) Proportion of influenza A and B isolations (by date of collection) among all adult patients' specimens that were submitted to the WHO reference laboratory at Queen Mary Hospital. C) Weekly influenza-like illness (ILI) (defined as fever plus cough or sore throat) consultation rates in sentinel networks of outpatient clinics in the private (GP) and public (GOPC) sectors. D) Weekly rates of public hospital admissions in young children ($\leq$4 years) with a principal diagnosis of influenza (International Classification of Diseases, 9th revision, code 487), where the denominator is the general population of the same age. E) Daily estimates of the effective reproductive number based on the laboratory and sentinel outpatient data. Source for panels B–D: (*7*).

We emphasize that our results must be interpreted with caution; in particular, influenza might have continued to circulate for a longer period had the school closures not been implemented. Furthermore, notwithstanding our tentative null findings, some previous reports have demonstrated that school closures may be effective at mitigating influenza seasons. For example, a study showed significant reduction in respiratory infections during school closures in Israel (*14*), and a recent model estimated that school holidays prevent 16%–18% of seasonal influenza cases in France (*12*).

We acknowledge that our assessment has the benefit of hindsight, whereas at the time the decision was made to close schools it might well have been unclear from surveillance data that the influenza season was only moderate and might have already been in natural decline. Although daily hospital admissions data were available in real time from a new integrated computer system and therefore did show the decline, this system only reflected serious illness. However, outpatient sentinel data, which are more indicative of overall influenza activity in the general community, were available with an ≈7-day lag; reports of laboratory reference data lagged even further. If public health decisions are to be made on the basis of prospective surveillance, these systems must be improved to reflect real-time or near real-time reporting and analysis. One possibility in Hong Kong would be to use the wealth of data from rapid influenza tests in hospitals, now that >1,000 rapid tests are conducted every month on most newly admitted patients with pneumonia or respiratory symptoms. Furthermore, although most local surveillance data are aggregated (Figure), the spread of influenza likely varies according to population subgroup. For example, influenza infections in children cause considerable illness and death, and it is often hypothesized that children are affected generally earlier in epidemics because of the higher transmission rates (*15*). Therefore, justification is strong for local authorities to begin collecting and

reporting timely age-specific community surveillance in sentinel and laboratory networks.

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Recent Shift in Age Pattern of Dengue Hemorrhagic Fever, Brazil

To the Editor: Brazil is responsible for >60% of reported cases of dengue fever (DF) in the American region (a designation of the Pan American Health Organization, which includes all of North, Central, and South America) (*1*). The epidemiologic characteristics of dengue diseases in Brazil differ from those described in Southeast Asia. In Brazil, the incidence of DF and dengue hemorrhagic fever (DHF) is highest in adults. By contrast, in Southeast Asia, DHF cases predominate and occur more often in children than in adults (*2*,*3*).

We describe a preliminary report of a shift in age group predominance that was observed during the 2007 countrywide dengue epidemic in Brazil. The Hospital Information System is the source of data describing the distribution of DHF cases from January 1998 through December 2007 (*4*). In Brazil almost all patients with a diagnosis of DHF are hospitalized. This country has promulgated the use of the World Health Organization's DHF case definitions (International Classification of Diseases, 10th revision – A91). In the 2007 epidemic, a larger than normal proportion of cases were DHF (2,706), more than twice the largest number of such cases reported in previous years. Moreover, in 2007 >53% of cases were in children <15 years of age; during 1998–2006, the predominance of DHF cases were in the 20- to 40-year age group (online Appendix Figure, available from www.cdc.gov/EID/content/14/10/1663-appF.htm). During 1998–2006, the percentage of DHF cases in children varied from 9.5% (in 1998) to 22.6% (in 2001).

Of the 2,706 DHF cases in 2007, 1,710 (63.2%) were reported from the northeast region; 1,119 (65.4%) of these were in children <15 years of age. The southeast region had the next largest number (558), accounting for 20.6% of all reported cases; however, only 26.2% were in children. Other regions with cases, central-west and northern, reported no substantial change in age distribution compared with earlier years. Among the 9 states in northeastern Brazil, DHF predominance in children was observed in Maranhao (609 cases; 92.0% in children), Rio Grande do Norte (97 cases and 77.6%), Pernambuco (316 cases and 67.0%), and Ceara (197 cases and 48.0%).

The change in age distribution of cases in 2007 is unique in the modern history of dengue in Brazil and requires an explanation. Dengue 1 and 2 viruses, which were introduced in the 1990s, generated epidemics of DF characterized by a low incidence of DHF, predominantly in adults. With the introduction of dengue 3 virus in 2000–2001, DF epidemics of greater magnitude were observed, with a slightly larger fraction of DHF cases. Differences in the epidemiologic patterns in Southeast Asia and the American region have been attributed to genetic resistance in black populations and to underreporting of DHF cases, among other factors (*2*). These factors seem insufficient to explain the sudden change observed; should it persist—as it has in Venezuela, Colombia, Central America, and Cuba—this change may bring dengue in Brazil to a pattern closer to that of Southeast Asia (*2*). This change in epidemiologic pattern of dengue cases supports calls for improvement in design of dengue surveillance studies to include, where possible, population-based serologic studies. These epidemiologic changes also serve as an alert to health authorities in the American region to update their healthcare services to provide agile, opportune, and good quality care for patients, particularly children, with DHF, to reduce deaths.

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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.

Confirmed *Mycoplasma pneumoniae* Endocarditis

To the Editor: In Rosario, Argentina, during June 2005, a 15-year-old boy was hospitalized because of a 2-month history of fever. The patient had no history of cardiac disease or intravenous drug use. The results of the physical examination and the laboratory tests were within normal limits, except for an increased leukocyte count (14,000/µL) with 68% neutrophils.

Transesophagic echocardiography showed mural vegetation on the right ventricle (30 mm × 20 mm) with no valve involvement. The patient was empirically treated with penicillin, gentamicin, and ceftriaxone. After treatment failed to produce a response, blood was submitted for culture for mycobacteria, brucellae, bartonellae, molds, and yeasts. BacT/ALERT bottles (bioMérieux, Durham, NC, USA), Hemoline performance biphasic medium (bioMérieux, Marcy L'Etoile, France), lysis centrifugation, and homemade culture broth were used. All culture results were negative. Results of PCR performed on serum for *Actinobacillus actinomycetemcomitans* were also negative. Because only the first samples were obtained before antimicrobial drug administration, a false-negative result was suspected. The patient underwent surgery for pulmonary microembolisms, and the vegetation was removed 4 weeks after drug treatment had started. The histologic appearance of the vegetation was consistent with infectious endocarditis, but the culture result was negative.

After 6 weeks of treatment, the patient was discharged from the hospital; however, 10 days after discharge he again became febrile and was readmitted to the hospital. The vegetation was again found. On this second admission, all cultures were performed before administration of antimicrobial drugs, and several types of culture media were used. In the absence of any growth by day 6, the patient's serum was screened for antibodies to *Mycoplasma pneumoniae, Chlamydia pneumoniae, and Bartonella henselae*. Serologic tests for immunoglobulin (Ig) G and IgM were conducted by indirect immunofluorescence assay (slides from Bion; Des Plaines, IL, USA) and fluorescein-labeled anti-human IgG and IgM (bioMérieux). For the IgM assay, the serum was pretreated with IgG/RF stripper (The Binding Site Ltd., Birmingham, UK). The titers for *M. pneumoniae* IgG and IgM antibodies were 2,048 and 160, respectively. Blood cultures were then subcultured in homemade Hayflick medium. These samples were incubated in 5% CO_2 in a 37°C incubator and examined 2×/week for typical *M. pneumoniae* colonies.

After 9 days of incubation, Hayflick agar plates inoculated with aliquots taken from homemade blood culture bottles (beef extract 5 g, yeast extract 5 g, peptone 10 g, glucose 2 g, NaCl 5 g, Na_2HPO_4 2.5 g, sodium heparin 10,000 U, distilled water to 1,000 mL, pH 7.6) showed colonies consistent with *M. pneumoniae.* No isolates were recovered from commercial blood culture bottles.

Result of hemolysis test with sheep blood was positive. The isolate was definitively identified as *M. pneumoniae* after P1 cytadhesin gene amplification by nested PCR, with primers P1-40, P1-178, P1-285, and P1-331 (*1*).

After mycoplasma were was isolated, intravenous clarithromycin was added to ceftriaxone; the ceftriaxone was discontinued 1 week later. The patient's clinical condition improved, and he was discharged 3 weeks after bacteriologic diagnosis with a treatment regimen of oral levofloxacin. After 6 months of treatment, the vegetation was reduced with no evidence of calcification.

Mycoplasma spp. have rarely been associated with endocarditis; until 2007, reports of only 8 cases had been published (*2–8*). The patient described herein had no underlying medical problems or immunodeficiency. Results of lymphocyte subsets, immunoglobulin titers, response to tetanus toxoid, and pneumococcal capsular polysaccharide were within reference ranges.

Cases of culture-negative endocarditis are not routinely investigated for mycoplasmas; however, the role of these microorganisms as a cause of endocarditis might be underestimated. *Mycoplasma* spp. cannot be detected by Gram stain and are difficult to isolate in bacteriologic culture media. Commercial blood culture broths that use sodium polyanetholsulfonate as an anticlotting agent are not suitable for growing these microorganisms (*9*). Other diagnostic approaches include the detection of specific DNA sequences or the use of broad-range eubacterial primers in cardiac tissue (*6*). In the patient reported here, the clinical sample (vegetation) was not available for diagnostic *M. pneumoniae* gene amplification. We failed to detect *M. pneumoniae* by PCR-mediated gene amplification directly from whole blood and plasma. Theoretically, specific PCR should be more sensitive than culture, as shown in respiratory specimens, but to date attempts to detect *M. pneumoniae* in blood by PCR have not been successful. The bacterial load in blood may have been too low to detect the amplified product by ethidium bromide–stained gel electrophoresis. The larger volume of blood used and the preincubation in broth with yeast extract for 7 days could have improved the recovery by culture. Another cause of reduced PCR sensitivity may have been the use of frozen samples.

This case of endocarditis caused by *M. pneumoniae* was confirmed by culture and occurred in a patient with no previous heart disease. Further studies are needed to evaluate the real incidence of *M. pneumoniae* as

a cause of endocarditis as well as the occurrence of mycoplasma bacteremia in the absence of underlying infection of the endocardium.

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Human Rhinovirus Group C Infection in Children with Lower Respiratory Tract Infection

To the Editor: Human rhinoviruses (HRVs), members of the family *Picornaviridae*, were first isolated in 1956 (*1*); to date, >100 serotypes have been identified on the basis of nucleotide sequence homologies. HRVs were previously divided into 2 genetic groups, HRV-A (n = 75) and HRV-B (n = 25). Recently, a putative new and distinct rhinovirus group, HRV-C, has been reportedly found in some patients with respiratory tract infections (RTIs) (*2–8*). To extend these initial findings and assess the pathogenicity of HRV-C, we investigated its prevalence as well as its clinical and molecular features in children with lower acute RTIs in Beijing, People's Republic of China.

From July through December 2007, nasopharyngeal aspirates were collected from 258 children (167 boys and 91 girls) who had lower acute RTIs at the time of their admission to Beijing Children's Hospital. The children were 1 month to 15 years of age (mean age 37 months, median age 10 months). Nucleic acids were extracted from clinical samples by using the NucliSens easyMAG platform (bioMérieux SA, Marcy L'Etoile, France). Each specimen was tested for the presence of common respiratory viruses: human parainfluenza viruses 1–4, influenza viruses, respiratory syncytial virus, enteroviruses, human coronaviruses (229E, NL63, HKU1, and OC43), metapneumovirus, adenoviruses, and bocaviruses. To study the prevalence of HRV-C, we designed a specific reverse transcription–PCR (RT-PCR) that generated a 330-bp PCR product encompassing a portion of the 5′-untranslated region, the full virus capsid protein (VP) 4 gene, and a portion of the VP2 gene of the HRV-C genome. (All primer sequences and protocols of these assays are available from J.W. upon request.)

This RT-PCR detected HRV-C in 14 patients (12 boys and 2 girls, 1 month to 13 years of age [mean age 19 months, median age 6 months]). In 6 of the 14 patients, HRV-C was the only virus detected, which suggests a direct correlation between HRV-C infection and lower acute RTIs. In the remaining 8 patients, other respiratory viruses were also detected. Respiratory syncytial virus, the most important cause of lower acute RTIs in children, was codetected in 7 of the HRV-C–positive patients, and human parainfluenza virus 3 was codetected in the other patient. Human coronavirus NL63 was codetected with respiratory syncytial virus in 1 HRV-C–positive patient.

HRV-C infection may be seasonal. This virus was detected during only 3 of the 6 months in which specimens were collected. Specifically, HRV-C was detected in samples collected in October (7/50), November (5/96), and December (2/8) but not in those collected in July (0/37), August (0/42), or September (0/25). In contrast, HRV-A and HRV-B were detected in

each month (data not shown). Indeed, from July through December, HRV-A and HRV-B viruses were detected in 34 and 12 patients, respectively. Notably, in October 2007, HRV-C was detected in 7 patients, while HRV-A and HRV–B were detected in 5 and 2 patients, respectively, which suggests that the cluster of cases of HRV infections during this month was caused mainly by HRV-C.

The 14 HRV-C–positive patients had a variety of other diseases including pneumonia (6/14), bronchopneumonia (4/14), and peribronchiolitis (3/14). The most common clinical findings were cough (14/14), fever (9/14), and abnormal breath sounds on auscultation (11/14). Radiographic results were available for 10 of the 14 HRV-C–positive patients, all of whom had increased lung markings or patchy shadows. Although 3 of the 14 patients required admission to the pediatric intensive care ward, their clinical outcomes were favorable.

Phylogenetic analysis showed that the 14 sequences obtained during this study (GenBank accession nos. EU687515–EU687528) together with previously reported sequences formed a novel group of rhinoviruses (Figure). Six sequences (BCH221, BCH264, BCH200, BCH341, BCH217, and BCH250) displayed high similarity to HRV C025 (EF582386) from Hong Kong (*4*); 2 sequences (BCH249 and BCH343) displayed high similarity to NAT083 (EF077264) from the United States (*3*) and *Picornaviridae* strain 06-646 (EU081811) from Germany (*5*). BCH242 was similar to *Picornaviridae* strain tu403 (EU081795) from Germany (*5*). The other 5 strains were homologous to strains from Australia (EU155152–EU155154, EU155158) (*2*,*7*). These findings suggest that, as in other countries (*5*), the HRV-C strains circulating in China are diverse. Although HRV-C strains belonging to different gene clusters cocirculate, some genetically close strains dominated during certain periods, e.g., the C025-like strain (6/14) was dominant during the study period. A similar distribution pattern is observed in epidemics of HRV-A and HRV-B (*9*,*10*).

In conclusion, HRV-C strains were detected in hospitalized children with lower acute RTIs in Beijing. Coinfections were common and complex, which indicates that the role of HRV-C in patients with multiple infections should be further investigated. Our findings provide additional evidence that HRV-C is spreading globally (*8*) and suggest that HRV-C infections should be considered a serious public health concern.

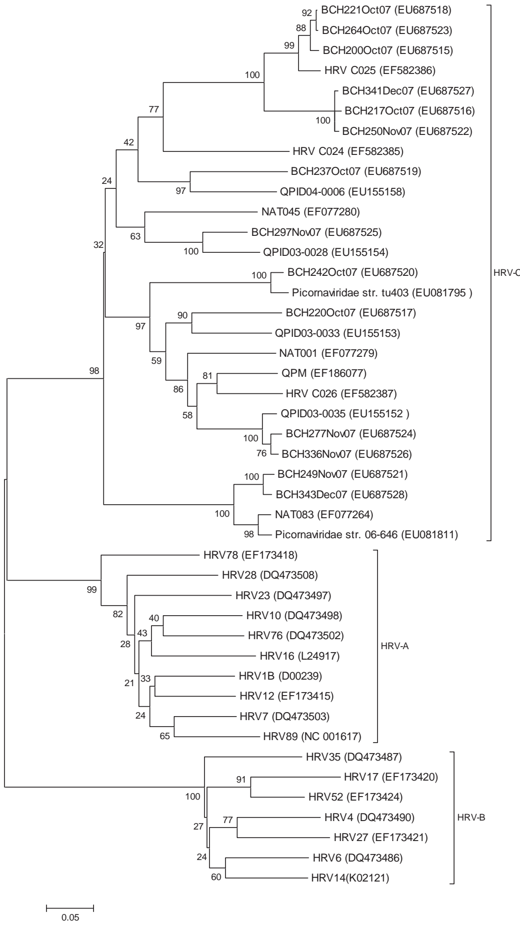

Figure. Phylogenetic analysis of the viruses detected in this study based on the nucleotide sequences of the virus capsid protein (VP)4/VP2 region. Using the VP4/VP2 nucleotide sequence (258 nt), we performed neighbor-joining analysis by applying the Kimura 2-parameter model in MEGA software version 4.0 (www.megasoftware.net). Bootstrap values from 1,000 replicates are shown next to the branches. The scale bar indicates evolutionary distance. Representative viruses from the different human respiratory virus (HRV) groups are included. GenBank accession numbers for reference sequences are indicated in parentheses.

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Serogroup A *Neisseria meningitidis* with Reduced Susceptibility to Ciprofloxacin

To the Editor: Reduced susceptibility to ciprofloxacin of *Neisseria meningitidis* has been reported with increasing frequency since 1992, mainly because of mutations in the quinolone resistance determining regions (QRDRs) of the gyrase and topoisomerase IV genes (*1,2*). Reduced fluoroquinolone susceptibility due to gyrase A mutations in serogroup A strains has previously been reported from a 2005 outbreak in Delhi, India (*1*). We describe 2 clinical isolates of serogroup A *N. meningitidis* with reduced ciprofloxacin susceptibility that were recognized in March 2003 and April 2006 in Israel, a country with low incidence of invasive meningococcal disease (<2/100,000/laboratory-confirmed cases/year) in which this serogroup accounts for <2% of cases (data from the National Center for Meningococci, Tel Hashomer, Israel).

The 2 isolates in question (M12/03 and M24/06; suffixes denote year of isolation) were compared with 2 fully susceptible strains, M44/01 and M23/00 (online Appendix Table, available from www.cdc.gov/EID/content/14/10/1667-appT.htm). MICs were measured by Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5% sheep blood. Demographic information was obtained from the Israel Ministry of Health Department of Epidemiology.

Chromosomal DNA was isolated by using the NucleoBond kit (Macherey-Nagel, Düren, Germany). The location of the QRDR in gyrase and topoisomerase IV genes was based upon prior studies in meningococci (online Appendix Table) and on the complete sequence of strain *N. meningitidis* Z2491 (serogroup A; GenBank accession no. NC_003116). We amplified and sequenced extended regions encompassing the QRDRs by using the upstream and downstream primer pairs in *gyrA* (522 bases) 5′-GTTCCGCGTCAAAATATGCT-3′, 5′-CCGAAATTGACGGTTTCTTC-3′; *gyrB* (649 bases) 5′-GGTTTGACCTGCGTGTTGTC-3′, 5′-CGGCTGGGCGATATAGATG-3′; *parC* (635 bases) 5′-CACTATGGTTTGCCGTTTTG-3′, 5′-ATTTCGGACAACAGCAATTC-3′; and *parE* (610 bases) 5′-GGACAGGATGGCGATTTTG-3′, 5′-CGTCAGCAACTTCATCAACC-3′. PCR was performed by using *Taq* DNA polymerase (New England BioLabs, Beverly, MA, USA). DNA sequencing was performed using the ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Screening for plasmid-mediated quinolone resistance genes was carried out by multiplex PCR amplification of *qnrA*, *qnrB*, and *qnrS* as

previously described (*3*). Multilocus sequence typing (MLST) was carried out by using the primers, protocols, and databases available from the Neisseria MLST website (http://pubmlst.org/neisseria) (*4*).

The online appendix Table shows our results and condenses previously published findings. The ciprofloxacin MIC for M24/06 was 42- to 125-fold higher than for susceptible strains and consistently 2-fold higher than that for M12/03. We have not referred to our isolates as resistant, because M12/03 would be categorized as "intermediate" by Clinical Laboratory Standards Institute breakpoints (*5*). The extended QRDRs in *gyrA* and *parC* of M44/01 (susceptible) were identical to those of *N. meningitidis* Z2491. M24/06 and M12/03 had a Thr91Ile mutation in *gyrA*. M24/06 also had Asn103Asp, Ile111Val, and Val120Ile mutations in *gyrA* (online Appendix Table; *1*). In M12/03, an Ala78Val mutation was found in *gyrA*, and new mutations Ile474Leu and Thr365Ala were found in *gyrB* and *parE*, respectively. No *parC* mutations were found.

Previous reports identified chromosomal mutations in *N. meningitidis* (online Appendix Table). M24/06 and M12/03 possess the same Thr91Ile mutation in *gyrA* as a 2002 serogroup B isolate from Spain (online Appendix Table) that had a similar increase in ciprofloxacin MIC (0.12 mg/L). The Thr91Ile mutation is homologous with the Ser83Leu mutation in *gyrA* of *Escherichia coli* that is responsible for a 60-fold increase in ciprofloxacin MICs (*6*). Further mutations in a primary target enzyme (gyrase) have been associated with additional 2-fold increases in the MIC of ciprofloxacin (*7*). The level of resistance observed in M24/06 might suggest additional mechanisms. An efflux pump mechanism is unlikely; we showed no reduction in MICs in the presence of reserpine (online Appendix Table) and this organism was fully susceptible to penicillin, tetracycline, erythromycin, and Triton X-100 (data not shown). This finding suggests the absence of an efflux pump encoded by a mutated *mtrRCDE* (*8*). Neither M24/06 nor M12/03 had plasmid-mediated genes *qnr* genes or elevated kanamycin MICs, suggesting the presence of *aac(6')-Ib-cr*. Both of these genes can confer low-level quinolone-resistance and facilitate the emergence of higher level resistance (*9*) (data not shown).

MLST showed that M24/06 and M12/03 did not derive from a single clone after selection of the T91I mutation. M12/03 was sequence type (ST) 2 and was isolated from a recent immigrant from Russia, which is the origin of most ST 2 strains deposited in the Neisseria MLST database (29/34 records; 85%). M24/06 was ST4789 in the ST5 clonal complex, isolated from a person who had immigrated many years previously from Romania. ST4789 has been encountered only once previously, in Dhaka, Bangladesh.

Disease associated with serogroup A *N. meningitidis* has been extremely unusual in Israel (*10*) and has remained rare. This serogroup comprised only 9 (1.9%) of all 463 isolates submitted during 1997–2006 (data from the National Center for Meningococci).

The isolates described in our study confirm that serogroup A should be added to the list of meningococci with the potential for reduced fluoroquinolone susceptibility and raise the question why they have appeared in a region with particularly low serogroup A meningococcal disease incidence while frequently encountered serogroups have remained fully susceptible. The importance of continuous monitoring for reduced ciprofloxacin susceptibility in these more prevalent serogroups has been emphasized by the recent replacement of rifampin by ciprofloxacin as the preferred agent for chemoprophylaxis of meningococcal disease in adults in Israel.

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Identification of All Dengue Serotypes in Nepal

To the Editor: Nepal is situated on the southern slopes of the Himalayas, surrounded by India on 3 sides and China to the north. Nepal's altitude ranges from 8,848 m in the Himalayas to 90 m in the Terai, the southern, low, flatland bordering India. Nepal is a disease-endemic area for many vector-borne diseases, including malaria, kala-azar, Japanese encephalitis, and lymphatic filariasis. Because of the porous border between Nepal and India, social, cultural, and economic activities in cross-border areas are common.

Dengue is an emerging disease in Nepal; presumably transmission is moving north from India into the Terai (*1*–*5*). The first report of dengue virus isolation or RNA (serotype 2 with nucleotide homology closest to a dengue virus type 2 isolate from India) was in 2008 involving a Japanese patient returning from Nepal in October 2004 (*5*). Entomologic investigations from the 1980s showed *Aedes albopictus* in the Terai plains, but *Ae. aegypti* has not been previously reported.

After Indian outbreaks now known to include all 4 dengue serotypes (*6*), a team from the Epidemiology and Disease Control Division, Kathmandu, investigated suspected cases of dengue fever during September–October 2006 in Banke, the district bordering Uttar Pradesh, India. The team collected blood samples from persons in Banke and, subsequently, from persons in a number of other districts and sent them to the National Public Health Laboratory in Kathmandu or the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand for analysis with ELISA, reverse transcription–PCR, (RT-PCR), or both.

Case definitions for dengue fever were adopted based on World Health Organization guidelines (*7*). Blood samples were obtained from patients with an acute febrile illness of 2–7 days' duration and with ≥2 of the following manifestations: headache, retro-orbital pain, muscular or joint pain, and rash. If laboratory tests were positive, cases were confirmed. Results were confirmed by ELISA performed at the Armed Forces Research Institute of Medical Sciences as previously described (*8*). Positive results were immunoglobulin (Ig) M ≥40 units or IgG ≥100 units. RT-PCR was performed by extracting RNA from 140 μL of each serum sample using QIAGEN Viral RNA Extraction Kit per manufacturer's instructions (QIAGEN, Germantown, MD, USA). RT-PCR and nested PCR were conducted according to the Lanciotti protocol (*9*) with the following modifications. Reverse transcriptase from avian myeloblastosis virus (Promega, Madison, WI, USA) was used in the first round RT-PCR. The concentrations of the primers used in the RT-PCR and nested reactions were reduced from 50 pmol to 12.5 pmol per reaction, and the number of nested PCR amplification cycles was increased to 25.

Serum specimens were obtained from 70 suspected case-patients from 16 districts from October 13 through December 3, 2006; 25 confirmed cases (13 by ELISA, 10 by RT-PCR, and 2 by both tests) came from 9 districts (Table). The average age was 29 years (range 5–65 years); 80% of the case-patients were men. Three patients had a history of travel to India, but clusters of dengue fever cases reported in October (Banke and Dang districts) indicated local transmission was occurring among patients with no travel history. The Terai districts accounted for 80% of cases. Entomologic collections done indoors and outside at 5 different sites reporting suspected cases identified *Ae. albopictus* and *Ae. aegypti* in all 5 districts.

These clinical and laboratory test results confirmed the presence of all 4 dengue serotypes. Notably, patients from the Dang district had no travel history outside the Dang valley. Because *Aedes* spp. have been identified in Dang, the data strongly suggest the existence of an endemic cycle of dengue. Underreporting is expected in the absence of diagnostic facilities at the field level. It is unclear whether the predominance of male patients is indicative of greater outdoor as opposed to indoor transmission. Of note, *Ae. albopictus* has been found in the country since the 1980s; in this study, we found *Ae. aegypti* in Nepal. Men typically wear short-sleeved clothes due to hot and humid conditions and, therefore, are frequently exposed to mosquito bites. However, men may also access the healthcare system more frequently. The ages of case-patients point to a relative lack of dengue immunity among the older population, and this finding is consistent with a new introduction of dengue. Because dengue hemorrhagic fever appears when >1 serotype becomes endemic to an area (*10*), the presence of all 4 serotypes portends the emergence of more severe dengue disease in Nepal.

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Table. Dengue laboratory test results, National Public Health Laboratory, Nepal, and AFRIMS, Bangkok, 2006*

Patient no.	Age, y	Gender	Residence	Travel history	ELISA	RT-PCR
1	39	M	Kathmandu	Unknown	Positive	DEN-3
2	48	M	Banke	Yes	Negative	DEN-3
3	18	M	Banke	No	Negative	DEN-3
4	20	M	Banke	No	Negative	DEN-3
5	22	M	Banke	No	Negative	DEN-3
6	25	M	Banke	No	Negative	DEN-3
7	25	M	Kathmandu	Unknown	Negative	DEN-1
8	26	F	Kathmandu	Unknown	Positive	DEN-3
9	38	M	Parsa	No	Negative	DEN-4
10	16	M	Dhading	Unknown	Negative	DEN-2
11	25	M	Jhapa	No	Positive	ND
12	37	F	Parsa	Unknown	Positive	ND
13	38	M	Dhading	No	Positive	ND
14	24	M	Banke	No	Positive	ND
15	36	M	Banke	No	Positive	ND
16	22	M	Parsa	Unknown	Positive	ND
17	5	F	Rupandehi	No	Positive	ND
18	13	M	Dang	No	Positive	ND
19	35	F	Parsa	No	Positive	ND
20	20	M	Kathmandu	Yes	Positive	ND
21	40	M	Kapilbastu	No	Positive	ND
22	20	F	Rupandehi	No	Positive	ND
23	42	M	Dang	No	Positive	ND
24	65	M	Banke	Yes	Positive	ND
25	28	M	Dang	No	Positive	ND

*AFRIMS, Armed Forces Research Institute of Medical Sciences; RT-PCR, reverse transcription–PCR; DEN, dengue; ND, not done.

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Detecting Avian Influenza Virus (H5N1) in Domestic Duck Feathers

To the Editor: Free-range domestic ducks can be a key factor in regional spreading of Asian subtype H5N1 avian influenza (AI) virus (*1–3*). Even asymptomatic domestic ducks can shed the virus continuously from the oral cavity and cloaca (*3–5*). Therefore, early detection of infected ducks that are shedding the virus would reduce the risk of spreading AI virus (H5N1) in a region where the virus has been endemic in domestic ducks. We previously reported that AI virus (H5N1) can replicate in feather epidermal cells in asymptomatic domestic ducks (*6*). Feathers are living tissues that are easily collectible from live birds with minimal damage. We now report the usefulness of feathers for virus detection in domestic ducks.

An experimental infection study was conducted with Japanese domestic ducks (*Anas platyrhynchos* var. *domestica*) and influenza A virus (H5N1) A/chicken/Miyazaki/K11/2007 as previously described (*6*). Three 4-week-old domestic ducks (a–c) were inoculated intranasally with 10^7 50% egg infectious doses (EID_{50}) of 0.1 mL. All experimental procedures were approved by the Ethics Committee of the National Institute of Animal Health in Japan.

Inoculated ducks did not show any clinical signs except for persistent corneal opacity on day 3 or later. We collected 3–5 contour feathers, plucked from the body, and 2 sets of oropharyngeal and cloacal swabs from each duck at 24-hour intervals from days 2 through 10 postinoculation (pi). Samples were examined by rapid tests, virus isolation, and reverse transcription–PCR (RT-PCR). Feathers were also examined by immunohistochemical testing.

On-site rapid tests were performed with a commercial kit, QuickVue Influenza A+B (Quidel Corp., San Diego, CA, USA), which can detect influenza virus nucleoprotein. The first set of swabs was used for rapid tests according to the manufacturer's instructions. We also tested 1–2 sticks of the feather calamus (≈15–30 mg per stick) for rapid tests (online Appendix Figure, panel A, available from www.cdc.gov/EID/content/14/10/1671-appF.htm). Briefly, we put the calamuses into the test tube containing attached reagent solution (340 µL) and chopped them into small pieces with an iris scissor and then placed the test strip in the tube. We obtained the following results: feathers tested positive for influenza A virus from days 3 through 6 pi in 1 duck (a), and on days 3 and 4 pi in 2 ducks (b and c), whereas all oropharyngeal and cloacal swabs were negative (online Appendix Figure, panel B).

For virus isolation, we used a second set of swabs placed in 1 mL of phosphate-buffered saline containing antimicrobial drugs and the remaining 2–3 feather calamuses. Virus titers of swabs and 10% (wt/vol) feather homogenate supernatants were calculated with 10-day-old embryonated chicken eggs and expressed as EID_{50}/mL. Viruses were isolated from the oropharyngeal swabs, cloacal swabs, and feathers of all birds, and feathers tested positive for the virus for a longer period than did the swabs (Table). Although the feather samples used for virus isolation differed from those used in rapid tests, the higher virus titers of feathers in each bird corresponded to the positive period of feathers for rapid tests.

One-step RT-PCR was performed on the total RNA extracted from the same samples as in virus isolation to detect the H5 AI virus gene (SuperScript One-Step RT-PCR System; Invitrogen, Carlsbad, CA, USA). The 1:10 dilution of RNA templates was used for feathers. The primers used were H5–248–270F and H5–671–647R; the expected product was 424 bp (*7*). The sensitivity of RT-PCR was slightly higher than that of virus isolation except for the results with cloacal swabs (Table).

Immunohistochemical testing was performed to detect influenza virus nucleoprotein in the feather tissue by using a rabbit polyclonal antibody

Table. Results of virus isolation and RT-PCR in 3 domestic ducks inoculated with influenza A virus (H5N1)*

	Virus titer (RT-PCR result)†								
	Duck a			Duck b			Duck c		
dpi	F	O	C	F	O	C	F	O	C
2	4.2 (+)	2.0 (+)	– (–)	1.7 (+)	1.7 (+)	– (+)	2.8 (+)	2.0 (+)	1.7 (–)
3	6.9 (+)	2.7 (+)	– (–)	4.5 (+)	3.0 (+)	2.5 (+)	4.8 (+)	1.7 (+)	2.8 (–)
4	6.8 (+)	3.7 (+)	1.7 (–)	2.3 (+)	3.5 (+)	1.7 (–)	4.8 (+)	3.3 (+)	– (–)
5	6.5 (+)	– (+)	– (–)	1.7 (+)	– (+)	– (–)	2.5 (+)	1.7 (+)	– (–)
6	5.6 (+)	– (+)	– (–)	– (–)	– (–)	– (–)	4.0 (+)	– (–)	– (–)
7	3.8 (+)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
8	5.8 (+)	– (–)	– (–)	– (–)	– (–)	– (–)	– (+)	– (–)	– (–)
9	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)
10	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)	– (–)

*RT-PCR, reverse transcription–PCR; dpi, days postinoculation; F, feathers; O, oropharyngeal swabs; C, cloacal swabs.
†Virus titer expressed as 50% egg infectious doses per mL; –, negative for virus isolation. RT-PCR result: +, positive; –, negative.

(ab22285; Abcam Ltd., Cambridge, UK). Virus antigens were detected in feather epidermal cells from days 3 through 6 pi, and in a few stromal cells in the feather pulp on days 3 and 4 pi (online Appendix Figure, panel C).

Our results indicate that larger amounts of viruses can be isolated for a longer time from feathers than from swabs. Therefore, feathers can be considered useful samples for surveillance or diagnostic examination of AI virus (H5N1) in domestic ducks. The epidermis, the outer layer of the feather, is a tissue that has poor host immune response against viral replication (*8*). As has been observed in virus isolation, viruses may be able to survive longer in differentiated epidermal tissue such as contour feathers.

The sensitivity of the rapid test was not adequate for swabs, a finding similar to that of other studies (*9*,*10*). However, positive results for rapid tests of feather samples only may shed light on the on-site field detection of AI (H5N1) in asymptomatic domestic ducks. When virus shedding from domestic ducks is maintained at a low level of viral load during the infection, selecting the sample with higher viral load and antigens in tissues, such as feathers, can increase the detection rate obtained from on-site examination. Our results show the potential of feathers as candidates for early AI virus (H5N1) detection.

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Neisseria gonorrhoeae Meningitis in Pregnant Adolescent

To the Editor: Dissemination is a rare complication of gonococcal infection and has been observed in 0.5%–3% of patients (*1*). We describe a new case in a pregnant adolescent infected with a strain resistant to ciprofloxacin and tetracycline.

A 14-year-old girl of Ecuadorian descent, 24 weeks pregnant, sought treatment after a 48-hour history of holocrania cephalea, fever, nausea, and vomiting. She had returned a few days before from a holiday trip to Quito, Ecuador, and had not taken any antimicrobial drugs. She was admitted to hospital with a temperature of 40ºC and neck stiffness. Results of a neurologic examination were otherwise normal.

Laboratory blood tests at hospital admission showed a leukocyte count of 13,400 cells/mm^3 (with a mature neutrophil count of 87%), hemoglobin of 10.8 g/dL, and a platelet count of 611,000 cells/mm^3; electrolyte levels and results of liver function tests were normal, but a total cholesterol count

of 267 mg/dL and a triglyceride level of 440 mg/dL were found. The level of C-reactive protein was elevated (17.90 mg/dL). Coagulation factors were within normal values, with the exception of a fibrinogen value of 917 mg/dL. Levels of complement components C2, C3, C6, C7, C8, and H factor were greatly elevated. The cerebrospinal fluid (CSF) sample obtained in the casualty ward had a leukocyte count of 5,000 cells/mm^3 (90% neutrophils, 10% lymphocytes), with glucose and protein levels of 20 mg/dL and 207 mg/dL, respectively. The patient received a 2-week course of intravenous cefotaxime (2 g every 4 h) and recovered without sequelae.

Gram smear of the CSF sediment was consistent with purulent meningitis. Culture on chocolate agar showed scarce growth of small, colorless mucoid colonies that showed positive reactions in oxidase and catalase tests. Gram stain of colony smears showed gram-negative diplococci and the API NH system (bioMérieux, Marcy l'Etoile, France) classified the bacterium as *Neisseria gonorrhoeae* (with a score of 87%). The result of the Phadebact Monoclonal GC test (Boule Diagnostics, Boule, Sweden) was positive. Cultures of endocervix, rectal, and pharyngeal samples obtained before treatment were negative. The strain was β-lactamase negative by chromogenic cephalosporin method (BBL Cefinase, Cockeysville, MD, USA) and classified as serogroup IA *rst* by agglutination with specific antisera (Neisseria Reference Center, Majadahonda, Madrid, Spain). The strain auxogroup, determined in modified Heckels media, (*2*) was arginine-hypoxanthine-uracil (AHU). Plasmid profile was obtained by standard alkaline DNA extraction (*3*) and showed a plasmid of ≈4.7 Md. The isolate was susceptible to penicillin (0.12 µg/mL) and cefotaxime (0.050 µg/mL) and resistant to tetracycline (16 µg/mL) and ciprofloxacin (4 µg/mL) by the agar dilution method (*4*).

Final identification of the bacterium was achieved by sequencing the 16S rRNA gene. The primers pA 5′-AGAGTTTGATCCTGGCTCAG-3′ and pH* 5′-AAGGAGGTGATCCAGCCGCA-3′ (Sigma-Genosys, Haverhill, England), as described by Edwards et al. (*5*), were used for amplification of 16S rRNA sequences. The PCR product was directly sequenced, and the nucleotide sequence matched the 16S rRNA gene of *N. gonorrhoeae* deposited in databases. The partial sequence of the 16S rRNA gene of *N. gonorrhoeae* determined in this study has been deposited in the European Molecular Biology Laboratory database under accession no. AM921674.

Neurologic manifestations of gonorrhea were observed as early as 1805 (*6*). However, the first well-documented case of gonococcal meningitis was not reported until 1922 (*7*) and so far, only ≈24 cases have been reported since 1922. In the preantimicrobial drug era, disemminated gonococcal infection (DGI) predominantly affected men (78%) but now is seen most frequently in women (97%) (*1*).

Other studies have listed factors that may facilitate spread of asymptomatic gonococcal infection, such as pregnancy (*1,8*), menstruation, viral hepatitis, differences in virulence between strains of gonococci, and host immunologic differences. The role of immunosuppressive conditions such as alcoholism or pregnancy remains unclear (*9*).

Knapp and Holmes (*10*) reported that 89% of *N. gonorrhoeae* isolated from patients with DGI were AHU auxotypes; proline dependence was also associated with DGI. These isolates were prevalent in Scandinavia and areas of the United States (Pacific Northwest, Minnesota, Wisconsin) with large Scandinavian-heritage populations. Isolated frequently in the 1970s, these strains are rarely isolated now. In other geographic areas, particularly South America, isolation of this type of strain is unusual.

In the past, all isolates recovered from patients with meningitis were susceptible to penicillin and tetracycline. Strains highly susceptible to penicillin generally require AHU for growth (*10*). Nevertheless, in the antimicrobial drug era, an increase in strains with an intermediate susceptibility to penicillin and a less invasive nature has been detected. More recently, penicillin-resistant strains recovered from patients with DGI and arthritis have emerged.

Gonococcal meningitis is a milder disease than meningitis caused by the pneumococcus or meningococcus because 6 of 9 patients seen before 1938 who were not treated with antimicrobial drugs survived (*10*) with no reported relapses or major neurologic sequelae, as in this case. In summary, we report an infrequent case of gonococcal meningitis in a pregnant adolescent with good clinical evolution and without sequelae or complications in her pregnancy.

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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.

Echinococcoses and Tibetan Communities

To the Editor: The People's Republic of China accounts for >500,000 cases of echinococcosis and more disability-associated life years (DALYs) lost because of this disease than any other world region (*1*,2). Hydatid cysts of *Echinococcus granulosus* (cystic echinococcosis [CE]), or the more pathogenic lesions with multiple vesicles caused by *E. multilocularis* infection (alveolar echinococcosis [AE]), usually grow slowly in the liver, so that severe illness and death may eventually occur in a high proportion of those with untreated infections (*3,4*). Apart from surgery, long-term anthelminthic therapy (>6 months) with the benzimidazole compound albendazole, although parasitostatic only, has a beneficial outcome in >50% of cases (*5*). To control the transmission of this zoonosis, veterinary public health measures must be emphasized (*6*).

In 2004 the Chinese Ministry of Health (MoH) undertook a nationwide assessment of 8 parasitic diseases, including malaria, schistosomiasis, and echinococcosis. To identify echinococcosis, 7 provincial MoHs carried out a mass abdominal screening of 34,500 persons using portable ultrasound scanners. The overall prevalence (2.5%) was highest in Tibetan communities in the Tibet Autonomous Region and in northwestern Sichuan and Qinghai Provinces (these latter regions form part of the eastern Tibetan Plateau). Collaborative studies involving the Sichuan Center for Disease Control and Prevention (based in Chengdu) and an international consortium of research institutes partly funded by the US National Institutes of Health (Bethesda, MD, USA) have shown an increasingly serious public health problem at the village, township, and county levels. In Shiqu County of Ganze Tibetan Autonomous Prefecture, 414 (12.9%) of nearly 3,199 persons surveyed by ultrasound (with serologic confirmation) exhibited CE or AE, including 19% in this category (*7*). The effects of human echinococcosis are substantial, with >50,000 DALYs lost in a population of 63,000 in Shiqu County (*8*).

Despite increased urbanization in China, >70% of Tibetans still live as seminomadic pastoralists on the high grasslands at an altitude >3,500 m. Most Tibetan herdsmen families keep at least 1 dog, and large numbers of ownerless stray dogs are tolerated by pastoralists and Buddhist monks. Risk factors for human echinococcosis (both CE and AE) in Tibetan communities usually include occupation, age (older persons are at higher risk), gender (higher risk for female), environment (pastoral landscapes), livestock ownership, and a history of dog ownership, as well as indicators of low socioeconomic status, including poor water quality and illiteracy (*7*,*9*) The prevalence levels of human AE in Ganze Tibetan Autonomous Prefecture (Sichuan Province) are among the highest recorded anywhere in the world. This situation presents a formidable challenge for early diagnosis, optimal affordable treatment, and prevention and control. Markham Hospital in Aba Tibetan Autonomous Prefecture (Sichuan) performed 1,200 operations for echinococcosis from 1992 through 2005, 20% for AE disease. For remote, high-altitude, pastoral Tibetan communities, however, long-term albendazole therapy is the only realistic treatment option, but regular follow-up of patients is difficult in these poorly accessible communities.

To address the public health concerns and consider options for controlling hydatidosis/echinococcosis in the eastern Tibetan Plateau, an International Workshop on Treatment, Prevention and Control of Echinococcosis was held in Chengdu in May 2006

with support from the Sichuan Center for Disease Control and Prevention, MoH Beijing, the New Zealand International Aid and Development Agency [NZIAD]), Fogarty-National Institutes of Health (USA), Xinjiang Medical University, and the Boulder-Lhasa Sister City Project. Recommendations stressed the following public health needs: improved treatment centers within the known disease-endemic counties or prefectures for long-term follow-up of patients after surgery and chemotherapy for both CE and AE disease, a better understanding of the epidemiology and ecology of transmission, and planning for pilot control interventions against both CE and AE transmission. NZAID made a detailed report of the implementation and effects of a pilot echinococcosis control program (2000–2006) in Datangma County, Ganze Tibetan Autonomous Prefecture (Sichuan Province). Problems occurred chiefly because of poor intersectoral cooperation, difficult logistics, cultural antagonism, lack of participatory planning, difficult access, treatment of dogs (with praziquantel), vaccination of livestock with the new EG95 vaccine (*6*), and lack of adequate surveillance of dog and livestock infection levels. The report indicated how many of these difficulties could be overcome. Consequently, the People's Republic of China MoH and provincial disease control networks approved funding in July 2006 to initiate pilot intervention programs against echinococcosis in 17 Tibetan autonomous counties of northwest Sichuan. Control options initially focused on regular supervised dosing of owned dogs and stray dogs (with praziquantel) by local operatives from district disease control centers and on improving health education at primary healthcare levels. Surveillance relies on measuring regularly the degree of *Echinococcus* infection in dogs by using a coproantigen test. Also, a specific age cohort of schoolchildren is monitored by ultrasound and serologic testing each year to determine changes in the prevalence of the 2 diseases. Albendazole is provided free, and the cost of surgery for hydatid disease is also subsidized through the new National Rural Cooperative Medical Insurance System.

In addition to the major public health problem now being recognized for echinococcosis in Tibetan communities, their general health indices are low (higher prevalence of tuberculosis, bone diseases such as arthritis, and poorer health in general) because of living and working at altitudes >4,000 m, compared with those in most other areas of China. Access and outreach should be improved (in conjunction with animal health initiatives) (*10*) for effective delivery of treatment, vaccination, and health education packages to these largely scattered and marginalized pastoral communities.

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Resource Allocation during an Influenza Pandemic

To the Editor: Considerable progress has been made in the United Kingdom to prepare for an influenza pandemic. After public consultation, an updated national framework (*1*) was recently published, along with new guidance on ethics (*2*), surge capacity, and clinical prioritization (*3*).

As Paranthaman et al. pointed out (*4*), difficult ethical choices must be made during a pandemic. Therefore, the UK Committee on Ethical Aspects of Pandemic Influenza published the ethical framework (*2*) designed to assist with and support the ethical aspects of policy and clinical decision making during and after an influenza pandemic. The fundamental principle underpinning the ethical framework is equal concern and respect, and it is expected that this principle, supported by 7 others listed in the guidance, will be used by clinicians, managers, and healthcare planners to develop policies on clinical issues for use during a pandemic. It is recognized and acknowledged within the document that the weight of a given principle will vary according to the circumstance.

Equally relevant is the interim guidance on surge capacity and prioritization in health services (*3*), which sets out a framework for the health service response in the United Kingdom during a pandemic and which advocates the wider use of the clinical triage criteria described for critical care by Christian et al (*5*). The proposed use of clinical triage at the primary care/secondary care interface starts to address the issue raised by Paranthaman et al. of who should be admitted to a hospital. This guidance expands early UK guidelines on the management of influenza-like illness during a pandemic.

We agree with Paranthaman et al. that early surveillance data are needed to rapidly inform clinical care guidelines in a pandemic. Therefore, efforts are ongoing to increase the resilience of health surveillance data gathering systems in the United Kingdom and to develop clinical systems for specific use during a pandemic. At the onset of a pandemic, it is intended that data will be gathered on the first few hundred patients by using a modification of the Web-based avian influenza management system of the Health Protection Agency. These data will provide important virologic and epidemiologic information to characterize the pandemic virus and inform modeling assumptions to validate "now casting" or real-time mathematical models (*6*) being developed in the United Kingdom and Europe to estimate the likely spread and impact of the pandemic. Furthermore, pilot projects are in preparation to develop clinical data collection systems in secondary care to assess treatments and outcomes during a pandemic.

In conclusion, contingency decisions outside normal patient pathways will be needed; the UK guidance, based on current knowledge and understanding, will help clinicians make difficult decisions on patient prioritization, plan surge capacity, build resilience into existing surveillance systems, and develop new systems that seek to inform the best use of resources to deliver optimal clinical care during an influenza pandemic. These decisions will be revised and modified to reflect new developments in the science.

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In Response: We are grateful to Phin and Davies for providing an update on the pandemic flu planning situation in the United Kingdom (*1*) after publication of our letter (*2*). We agree with their emphasis that pandemic planning in the United Kindgom is in

many regards well developed in comparison with other countries. Many of the updates that they describe provide useful contributions but the final version of UK ethical guidance is more a general statement of principles than a practical guide. Unfortunately, it has pulled back from some of the more definite statements in the earlier consultation documents available at the time of our letter.

An important need for practical guidance for frontline and managerial staff on the ethical aspects of pandemic response remains as a gap in the United Kingdom and elsewhere. For critical care, a useful recent contribution is the report produced by the Task Force on Mass Critical Care, especially the framework for allocation of scarce resources in mass critical care (*3*). The shift away from pandemic influenza–specific criteria to a more generic framework for resource allocation that can compare patients with pandemic influenza and those with other usual noninfluenza conditions is welcome.

Similar practical approaches outside the critical care area are important priorities for work in pandemic preparedness. In addition, systems must be in place for rapid modification and communication of the criteria for referral and admission, taking into account the severity of the pandemic and availability of healthcare resources. Healthcare services must not only be transparent and fair in this process, they must also be seen by the public as being so. Engagement with the public on values and principles underlying resource allocation would be of paramount importance during a pandemic.

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Risky Trade: Infectious Disease in the Era of Global Trade

Ann Marie Kimball

Ashgate Press, Aldershot, UK, 2006
ISBN-10: 0754642968
ISBN-13: 978-0754642961
Pages: 212; Price: US $59.95

The linkages between facets of globalization and the emergence and recrudescence of infectious disease are a topic of increasing concern for providers of public health, international business concerns, economists, and political elites. Such interdisciplinary inquiry is rare, and sorely needed, at the dawn of the 21st century. Ann Marie Kimball's book is a welcome addition to those few inquiries that cross the disciplines of epidemiology, economics, and political science. Jargon free, and well written, the book is an excellent analysis of the consequences of globalization upon public health and the consequences of disease for international trade and economic productivity.

The term globalization is frequently used in modern discourse but often poorly defined. Typically, it refers to the movement of financial capital and trade goods. However, a nuanced understanding of globalization accounts for negative externalities such as human-induced environmental change and the emergence and recrudescence of infectious diseases. Kimball addresses these linkages in deft fashion and notes the often negative consequences of the complex interactions between the worlds of trade, ecology, public health, and politics. Despite the great need for interdisciplinary approaches to deal with concatenating global problems, barriers between scientific disciplines, a "silo mentality," persist and undercut our capacity to respond to emerging threats.

Kimball argues that globalization and its associated processes (crowding, poor sanitation, travel and trade, intensive food production practices, and ecologic change) all increase the threat of pathogen emergence. Her book explains the mechanisms by which ecologic change drives processes of pathogenic emergence, facilitates zoonotic transfers, induces mutation, and permits the globalization of antimicrobial drug resistance. Kimball also provides an illuminating analysis of the mechanics of microbial interdependence between the industrialized and nonindustrialized worlds. She argues that globalization has directly contributed to the emergence of pathogens such as the severe acute respiratory syndrome (SARS) coronavirus and is increasing the probability of pandemic influenza. In sum, she argues correctly, globalization is creating a new ecology of disease.

In the domain of trade and economics, Kimball provides an illuminating discussion of the global blood trade and its pivotal role in the emergence of the HIV/AIDS pandemic. Following that discussion, she provides a detailed analysis of the negative economic impact of infectious disease (e.g., SARS, influenza, cholera, bubonic plague) upon trade and economic productivity and notes the economic damage resulting from trade embargoes imposed upon countries that exhibit epidemic infections. Frequently, such embargoes are the result of uncertainty-induced fear and lack any empirical basis. Kimball also provides a useful overview and analysis of the "compulsory licensing" provisions, and the protocols for Trade-Related Aspects of Intellectual Property Rights of the World Trade Organization (WTO). In the event of a public health emergency, such protocols enable nations to develop lifesaving medicines that infringe upon the patents developed by others. Furthermore, she argues that the WTO does not effectively represent the interests of the nonindustrialized countries, particularly when the interests of international business conflict with the well-being of the indigent.

Kimball also does an excellent job of critiquing domestic and international forms of public health governance. She recognizes, accurately, that the state does indeed have a central role to play in pathogen surveillance, the judicious use of quarantine, and the provision of public goods such as healthcare. She discusses the interaction (and occasional tensions) between sovereign states and international organizations (e.g, the World Health Organization and WTO) and notes the limitations of international health regimes, such as the International Health Regulations, even in their recently revised form. Kimball discusses the perils induced by low levels of surveillance and containment capacity in the nonindustrialized countries, noting that serious outbreaks of contagion frequently overwhelm local health infrastructures and health providers. Consequently, she stresses the need to bolster global pathogen surveillance, diagnostic, and response networks. Unfortunately, as Kimball duly notes, public health remains rather marginalized in the conduct of international politics and in the study of international relations as well.

Finally, the author provides an excellent critique of health governance at the domestic level within the United States. She begins with a discussion of protocols for domestic biodefense and briefly analyzes the utility of exercises such as Global Mercury, Dark Winter, and Top Officials (TOPOFF). Kimball questions the efficacy of the National Pharmaceutical Stockpile and notes the vulnerability of the US food supply. Further, she notes the lack of autonomy (and often capacity) of those divisions of the US bureaucracy tasked with the protection of public health, particularly the US Department of Agriculture. Kimball astutely warns of the perils of the self-congratulatory

"happy talk" so prevalent in international organizations, national governments, and nongovernmental organizations, as it leads to overestimation of response capabilities. Finally, Kimball warns that the lack of universal health insurance in the United States actually increases societal vulnerability to contagion.

In sum, this is a very good book, well-suited to public health practitioners and medical personnel, and senior undergraduates. And, frankly, it should be read by those in the realms of business and politics as well.

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The Making of a Tropical Disease: A Short History of Malaria

Randall M. Packard

Johns Hopkins University Press, Baltimore, Maryland, USA, 2007
ISBN: 978-0-8018-8712-3
Pages: 320; Price: US $24.95

This publication is one of a series published by the Johns Hopkins University Press on biographies of disease. Earlier volumes were Mania: A Short History of Bipolar Disorder and Dropsy, Dialysis, and Transplant: A Short History of Failing Kidneys. Malaria is clearly a worthy subject in this ambitious series.

The preface sets the stage for the treatise on malaria and establishes the author's interest and qualifications for writing the book. The first chapter provides a reasonable scenario for establishing Africa as the place of origin of human malaria parasites and the probable movement of the organisms with movements of early humans from Africa through southern Asia and eventually to the Pacific Islands.

The reviewer was somewhat uncomfortable with the complete absence of any discussion of the evolution of *Plasmodium* species in nonhuman primates because those parasites are clearly closely related to those found in humans. The statement that there are 4 species of malaria parasites that infect humans is inaccurate. Recent reports of the extensive occurrence of natural human infections with *P. knowlesi* in Borneo and the Philippines are an issue that warrants attention. (Experimental infections in humans with malaria parasites from nonhuman primates in Asia do not need to be detailed here.) Fortunately, some weaknesses in the discussion of the evolution of primate malaria parasites do not seriously detract from the detailed and well-written story of malaria as a human disease.

The movement of malaria into northern areas and its eventual retreat back to the tropics is well told and clearly addresses the central theme of "the making of a tropical disease." The discussions of the long history of malaria control efforts directed toward the vector and, to a lesser extent, the parasite without what the author considers adequate attention to the social aspects of malaria occurrence are well structured. The recounting of the disastrous Global Malaria Eradication effort is must reading for anyone interested in human malaria. The reviewer experienced this effort personally but continues to be fascinated with this extraordinary story.

The discussion of the current program, "Roll Back Malaria," is an essential part of this story. This ongoing and massive effort to bring malaria under control is multifaceted and heavily funded, and its eventual outcome may well inspire Dr. Packard to write an addendum to this interesting book.

The author's focus on poverty and its contribution to the continued presence of malaria in endemic areas, especially Africa, is well presented. There is no doubt that war, famine, political upheaval, and human poverty are primary issues in the continued presence of malaria as a major cause of illness and death. Unfortunately, this book does not offer a solution to these issues. This book should be read by and on the shelf of anyone working in or generally interested in the place of malaria in human history.

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Eric Mack (b. 1976) APMR-41553 (2007) Mixed media on canvas (182.8 cm × 91.4 cm) Used with permission of the artist. Photo by Fay Gold Gallery, Atlanta, Georgia, USA. www.erickmack.com

Collage and Assemblage in the Microbial World

Polyxeni Potter

"There are two problems in painting" said American artist Frank Stella (b. 1936) when he was still a brash young man. "One is to find out what painting is, and the other is to find out how to make a painting" (*1*). Stella's interest in the definition of painting may have reflected late 19th- early 20th-century modernist concerns. More contemporary concerns focus primarily on how to make a painting, how to use materials, methods, concepts, or traditions to create a work that may not even be called a painting. *How* a work is determines *what* it is (*2*). Atlanta artist Eric Mack, a man of his times, is part of the contemporary scene and its pressures to create a painting.

At one time, art was organized by schools, later by movements. Today, these designations are difficult to apply. Painting cannot be categorized exclusively as a certain palette or style, and no consistent look or theme can be used to quantify its sum. The old labels (abstract, realist, symbolist, surrealist, expressionist, narrative) may still describe some works. But others defy all convention. They have no images, no drawing, no color, no canvas, or are not made by the artist's hand.

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"I'm all about shape, pattern, and repetition of form," is how Mack describes his own work (*3*). Among his techniques, collage and assemblage are suited for discovering new forms and forming new ways to connect objects retrieved from the world. His canvases contain synthetic netting, natural fibers, leaves, Japanese text, board game graphics, corporate logos, small levers and sockets, plugs, dials and switches. These bits and pieces of objects, punctuated by a painted eyeball here, a peacock feather there, are laboriously integrated into a fluid backdrop of color applied by brush or aerosol. The effect refers to recent traditions, among them abstract expressionism, pop, and graffiti (*4*).

"She got me those clippers and told me that once I learned to cut hair," Mack says of his mother, a cosmetologist, "I would never have to worry about going hungry." His career as barber flourished throughout his years at the Atlanta College of Art, when he was co-owner of "Barber's Edge" on Buford Highway, the city's bustling multiethnic corridor where he tended all styles and customers: "Flat-

tops, fades, skin fades, shadow fades, babies to old people, black, white, Chinese, Mexican, Puerto Ricans, Cubans, Africans, anyone who has hair." But his artistic career started much earlier, "I remember different breakdancing moves like the Windmill that you could do. I'd draw people doing the dance moves and color them up and sell them for, like, a dollar or two dollars in elementary school" (*5*).

Mack's American childhood, begun with his birth in suburban Charleston, South Carolina, was otherwise unremarkable. Yearly trips to New York to visit family exposed him to music, which would become a major inspiration. As an art student, he met and was influenced by another Atlanta artist, Charles Nelson, who encouraged him to move from illustration to painting. "I was too intimidated by the brush. Once I took a chance and tried it out, I thought 'This brush is like the clippers.' It came like I had been doing it for a long time" (*5*). He joined a group of local artists, among them Kevin Cole and Kojo Griffin, exhibited his work often, and became a fixture of the Atlanta art scene.

APMR-41553 on this month's cover of Emerging Infectious Diseases embodies the sprawl, clutter, and flux of today's human communities. A complex composite of unlikely pieces, it portrays new forms constructed out of old and attached as never before.

An organic arrangement of viable human, mechanical, and environmental parts, the work has rich metaphorical potential. The solid microcosm anchored against undulating fluid, now blotted with clumps of color, now disappearing against bare canvas, encompasses all, including human and microbial interaction.

"Our relationship to infectious pathogens," wrote the late Joshua Lederberg, "is part of an evolutionary drama. Here we are. Here are the bugs" (*6*). These ubiquitous bugs have, among other advantages, the ability to change in ways that make them dangerous. For example *E. coli* O157:H7 started out as a pathogen capable of causing mild diarrhea. Newly acquired genes transformed it into a virulent microbe that also destroys kidneys and red blood cells (*7*). Diversification in some strains of group A streptococci, common bacteria that normally do not cause disease in humans, is causing the reemergence of a severe form of invasive disease (*8*).

Collage and assemblage, which work so well in Mack's art, mirror microbial activity that can cause havoc in the global community. Like the artist's bits of objects, microbial ultrastructures can reassort, recombine, and reassemble into brand new entities. They adapt to new ecologic niches or species, produce new toxins, and bypass or suppress immune defenses to infect humans and animals. Their plasticity frustrates vaccine development, and they become resistant to even the most potent drugs.

For all its apparent fragmentation, Mack's vision is universal and positive. The reassembled objects form a new working composite. But the microbial equivalent is still a puzzle seeking solution in science's mixed media: new technologic bits and pieces (vaccines, drugs, diagnostic tools) arranged against crowding and social, political, and economic stratification.

Acknowledgment

The author thanks Louise E. Shaw for her help in obtaining permission to use APMR-41553.

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Framework for Leadership and Training of Biosafety Level 4 Laboratory Workers

Mixture for Controlling Insecticide-Resistant Malaria Vectors

Highly Pathogenic Avian Influenza (H5N1), Sub-Saharan Africa

Multidrug- and Extensively Drug–Resistant Tuberculosis, Germany

Multidrug-Resistant Tuberculosis among US-bound Hmong Refugees, Thailand

Molecular Epidemiology of MRSA, Rural Southwestern Alaska

Antimicrobial Drug Use and Resistance in Europe

Porcine Respiratory and Reproductive Syndrome Virus Variants

Changes in Incidence of Japanese Encephalitis Virus Genotype, Thailand

Successful Treatment of Disseminated *Acanthamoeba* sp. Infection with Miltefosine

Influenza Virus (H5N1) in Live Bird Markets and Food Markets, Thailand

Delinquent Mortgages, Neglected Swimming Pools, and West Nile Virus, California

pLDH Malaria Rapid Diagnostic Test to Identify *Plasmodium knowlesi* Infection

Tourism and Specific Risk Areas for *Cryptococcus gattii*, Vancouver Island, Canada

Metagenomic Diagnosis of Bacterial Infections

New Hepatitis B Virus Variant, Southeast Asia

Complete list of articles in the November issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

October 30–November 1, 2008
Ninth International Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases Meeting (MEEGID IX)
University of California at Irvine
Irvine, CA, USA
http://www.th.ird.fr/site_meegid/menu.htm

February 12–13, 2009
The International Symposium on the Asian Tiger Mosquito
Rutgers University
New Brunswick, NJ, USA
http://www.rci.rutgers.edu/~vbcenter/atmsymposium.php

February 13–16, 2009
International Meeting on Emerging Diseases and Surveillance (IMED 2009)
Hotel Hilton
Vienna, Austria
http://imed.isid.org

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Article Title

Microbial Interactions during Upper Respiratory Tract Infections

CME Questions

1. Which of the following bacteria was most commonly isolated from nasopharyngeal swabs in the current study?

A. *Staphylococcus aureus*
B. *Moraxella catarrhalis*
C. *Streptococcus pneumoniae*
D. *Haemophilus influenzae*

2. Which of the following associations between bacteria in the current study is most accurate?

A. Colonization with *H. influenzae* was positively associated with *S. pneumoniae* colonization
B. Colonization with *M. catarrhalis* was positively associated with *S. pneumoniae* colonization
C. Colonization with *S. pneumoniae* was positively associated with *M. catarrhalis* colonization
D. Colonization with *H. influenzae* and *M. catarrhalis* was positively associated with *S. pneumoniae* colonization

3. Which of the following variables was associated with a significant decrease in the rate of colonization with *S. pneumoniae*?

A. Antibiotic use in the past 7 days
B. Younger age
C. Up-to-date vaccination with pneumococcal vaccine (PCV7)
D. Breast-feeding

4. Day care promoted colonization with which of the following bacteria?

A. *S. aureus*
B. *M. catarrhalis*
C. *S. pneumoniae*
D. *H. influenzae*

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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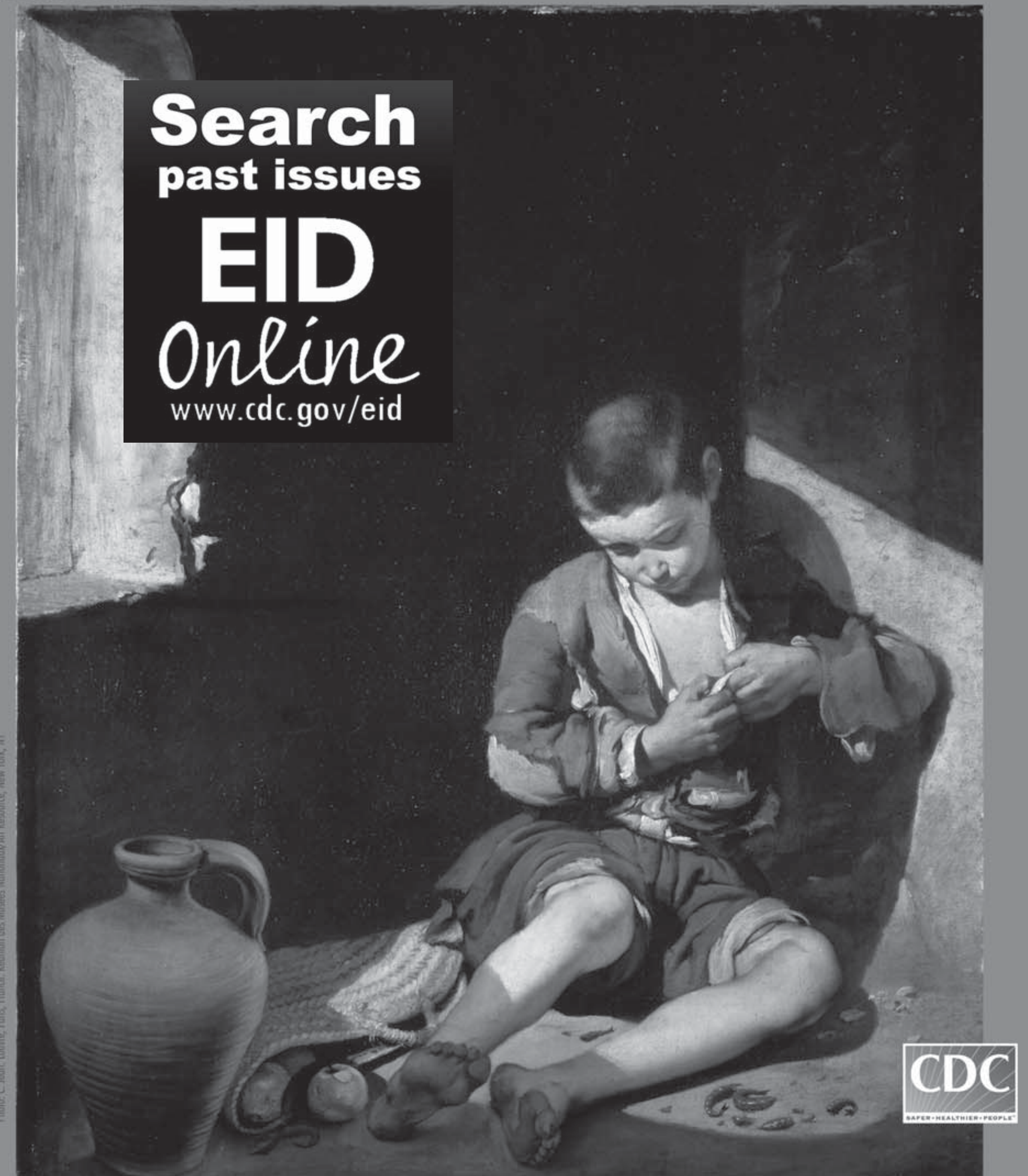

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