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On the Cover

Hale Aspacio Woodruff (1900–1980)
The Art of the Negro: Interchange (1950–1951)
Oil on canvas (360 cm × 360 cm)
Clark Atlanta University Collection of African-American Art,
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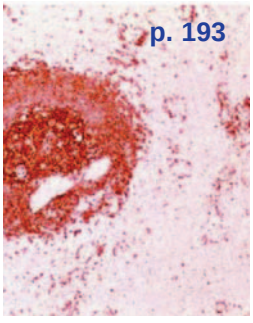
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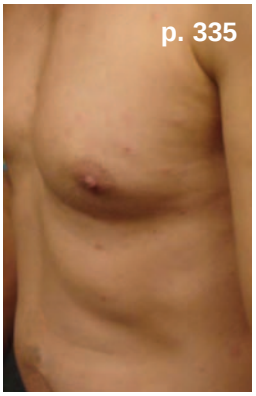

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Prevention of Immune Cell Apoptosis as Potential Therapeutic Strategy for Severe Infections

Janie Parrino,* Richard S. Hotchkiss,† and Mike Bray*

Some labile cell types whose numbers are normally controlled through programmed cell death are subject to markedly increased destruction during some severe infections. Lymphocytes, in particular, undergo massive and apparently unregulated apoptosis in human patients and laboratory animals with sepsis, potentially playing a major role in the severe immunosuppression that characterizes the terminal phase of fatal illness. Extensive lymphocyte apoptosis has also occurred in humans and animals infected with several exotic agents, including *Bacillus anthracis*, the cause of anthrax; *Yersinia pestis*, the cause of plague; and Ebola virus. Prevention of lymphocyte apoptosis, through either genetic modification of the host or treatment with specific inhibitors, markedly improves survival in murine sepsis models. These findings suggest that interventions aimed at reducing the extent of immune cell apoptosis could improve outcomes for a variety of severe human infections, including those caused by emerging pathogens and bioterrorism agents.

Despite success in controlling many infectious diseases, efforts to defend against the wide range of microbes that threaten human health continue to be challenged by the unexpected emergence of novel pathogens and possible use of a variety of virulent agents as biological weapons. A defensive strategy based solely on developing new vaccines and antimicrobial and antiviral drugs, each specific for only 1 or a few agents, is unlikely to be successful in dealing with potential microbial threats and will be exceedingly expensive. An alternative approach attempts to identify mechanisms shared by most or all severe infections that could be targets for pharmacologic intervention. Such generic therapies could supplement agent-specific treatment by increasing resistance to infection, potentially improving outcomes for patients in a variety of disease states.

One physiologic process that characterizes some severe infections is a massive loss of lymphocytes, dendritic cells, gastrointestial epithelial cells, and other cell types through apoptosis, or programmed cell death. This process is an apparent acceleration or dysregulation of the same process by which these cell populations are regulated during normal health (*1*,*2*). By impairing the development of adaptive immune responses needed for recovery, the apoptotic destruction of lymphocytes and dendritic cells could have a particularly adverse effect on disease outcome. Fortunately, because programmed cell death is an orderly biochemical process triggered by specific stimuli and executed by a limited range of enzymes, it could be inhibited through pharmacologic countermeasures, offering a novel approach to therapy.

We begin this article by summarizing evidence that a massive apoptotic loss of lymphocytes takes place in humans during the course of septic shock and describing similar findings in animal models of sepsis. Data are then presented that indicate that a marked die-off of lymphocytes also occurs in Ebola hemorrhagic fever, anthrax, and plague, which suggests that unregulated apoptosis of these cells is a component of many, and perhaps all, severe infectious processes and may contribute to high case fatality rates by impairing adaptive immune function. After describing encouraging results obtained in proof-of-concept tests of antiapoptotic interventions in lethal murine models of sepsis, we note some potential limitations of such therapy that could slow its introduction into the

*National Institutes of Health, Bethesda, Maryland, USA; and †Washington University School of Medicine, Saint Louis, Missouri, USA

therapeutic regimen. Whatever the potential role of such strategies, improved understanding of the causes, time course, and extent of programmed cell death will aid management of patients with severe infections.

Mechanism and Regulation of Apoptosis

Apoptosis, or programmed cell death, is the method by which tissue remodeling takes place during normal growth and development and the physiologic mechanism by which labile cell populations such as gastrointestinal epithelial cells, lymphocytes, dendritic cells, and neutrophils are regulated. Apoptosis is of particular importance for the immune system as the means by which self-recognizing lymphocytes are deleted and expanded lymphocyte populations are reduced at the conclusion of an acute immune response (*3*). This closely regulated, energy-requiring process can be initiated through 2 different mechanisms, each based on the successive activation of preexisting but dormant cysteine-aspartate proteases, or caspases (Figure 1).

As its name implies, the intrinsic apoptotic pathway begins within the cell, when toxic alterations bring about a decrease in mitochondrial transmembrane potential, leading to the opening of mitochondrial membrane pores and the release of cytochrome C and other substances into the cytoplasm. The extrinsic pathway, by contrast, is triggered by extracellular events through the binding to cell surface receptors of tumor necrosis factor (TNF) superfamily death ligands, including TNF-α and Fas ligand. Although the intrinsic pathway involves early activation of caspase-9, and the extrinsic pathway is mediated through caspase-8, both lead to activation of the "executioner" caspase-3 and a variety of proteases and endonucleases. Once begun, apoptosis may be described as an orderly disassembly of the cell from within. Chromosomal DNA is cleaved into oligonucleosomal segments, the nucleus is divided into discrete subunits, and the cell itself is partitioned into multiple membrane-bound fragments whose outer surfaces are marked by large numbers of phosphatidylserine molecules, leading to their rapid uptake by phagocytes. Because all multicellular organisms use programmed cell death to maintain and modify their tissues, this process does not evoke an inflammatory response, and its end products actually serve as antiinflammatory stimuli. Apoptosis thus differs markedly from necrosis, the chaotic breakdown resulting from trauma and other types of damage, in its morphologic and immunologic features (Table 1). Necrosis is characterized by the early loss of outer membrane function, rapid cytoplasmic swelling and disintegration, and release of cell contents into surrounding tissues, which evoke an intense inflammatory response.

A large number of cell-surface and cytoplasmic proteins participate in the detection and processing of signals that tip the balance toward or away from programmed cell death. These include members of the Bcl-2 protein family, which have both proapoptotic and antiapoptotic activity (Bcl-2 is antiapoptotic), and other inhibitors (Figure 1). Despite these elaborate control mechanisms, innate or acquired defects in the control of apoptosis may lead to a variety of disease states. For example, excessive inhibition of apoptosis is an underlying mechanism of cancer, while an inappropriate increase is seen in some neurodegenerative diseases and other conditions.

Lymphocyte Apoptosis in Sepsis

During normal health, the immediate fate of each lymphocyte is determined through continuous summation of a stream of proapoptotic and antiapoptotic signals that arrive from its external environment and from its internal cytoplasmic milieu. A shift toward initiation of apoptosis should therefore be expected during the early phase of sepsis, when bacteria or their byproducts stimulate macrophages to release proapoptotic substances such as TNF-α, nitric oxide, and glucocorticoids. As the disease develops, accumulating products of lymphocyte apoptosis can act as antiinflammatory stimuli, which contribute

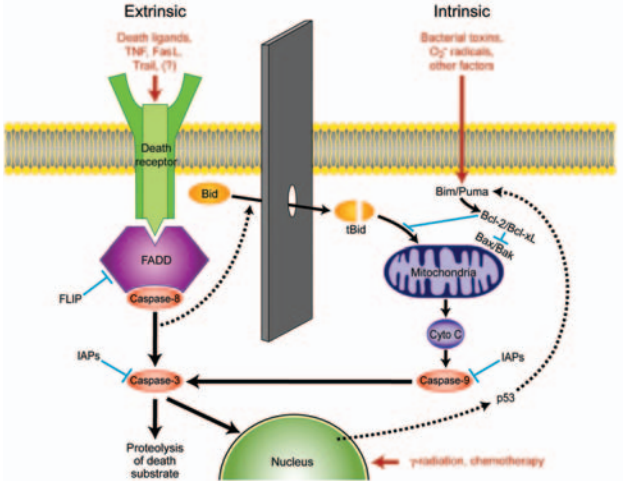

Figure 1. Apoptotic pathways of cell death. The extrinsic pathway is mediated by a variety of death receptor ligands, including tumor necrosis factor (TNF) and Fas ligand (FasL), that trigger apoptosis by binding to cell surface receptors. In the intrinsic pathway, several adverse factors act upon mitochondria to cause loss of the mitochondrial membrane potential, resulting in leakage into the cytosol of cytochrome C (Cyto C), which together with apoptotic protease activating factor 1 forms the apoptosome that activates caspase-9. Communication between the pathways exists through cleavage of Bcl-2 interacting domain (Bid) by active caspase-8 to form truncated Bid (tBid). Inhibitors of apoptosis (IAPs) can prevent caspase activation under certain conditions. Trail, tumor necrosis factor-α–related apoptosis-inducing ligand; Bim/Puma, Bcl-2 interacting mediator of cell death/p53-upregulated modulator of apoptosis; FADD, Fas-associated death domain; FLIP, Fas-associated death domain-like interleukin-1 converting enzyme-like inhibitory protein.

Table 1. Distinguishing features of apoptosis and necrosis

Feature	Apoptosis	Necrosis
General description	Genetically programmed, orderly process of cell death	Accidental cell death caused by acute injury or other exogenous effect
Membrane integrity	Preserved until late in cell breakdown process	Early loss results in cell and organelle swelling and rupture
Chromosomal DNA	Cleavage at nucleosomes produces ladder pattern on an agarose gel	Random fragmentation produces smear pattern
Inflammatory response	None; products have antiinflammatory effect	Release of intracellular contents causes acute inflammatory response

to the immunosuppression commonly observed as sepsis progresses to septic shock, and which can lead to a state of immune paralysis before death (*2*,*3*).

Numerous studies have demonstrated a massive apoptotic loss of lymphocytes during sepsis. A prospective investigation in adult patients compared spleens obtained either intraoperatively or within 6 hours after death from sepsis or trauma and found that those from sepsis patients showed a marked decrease in B cells and CD4 T cells (Figure 2) (*1*). The degree of splenic B-cell depletion corresponded with the duration of sepsis. Active caspase-9 was present in splenic lymphocytes with apoptotic features, suggesting a mitochondrial-mediated pathway of cell death, although evidence indicates that apoptotic cell death in patients with sepsis can also proceed by the death receptor pathway (*4*). In most patients, loss of cells from the spleen corresponded with a premortem decrease in circulating lymphocytes.

These findings were closely paralleled in another postmortem study, which showed that B and T cells and dendritic cells were markedly depleted in lymphoid organs of children dying of sepsis and that >3% of cells exhibited histologic signs of apopotosis (*5*). Approximately 15% of patients had prolonged lymphopenia during their terminal course. This report suggested a possible stimulus for apoptosis, in the form of persistent hypoprolactinemia, because prolactin up-regulates expression of the antiapoptotic protein Bcl-2. A third study also noted a profound loss of B and T cells in the spleens of neonates who died of sepsis and chorioamnionitis. Another study compared premortem blood counts in patients with septic shock, sepsis without shock, or nonseptic critical illness and found that increased lymphocyte apoptosis began early in septic shock, and that severe lymphopenia was predictive of a fatal outcome (*6*,*7*).

Extensive loss of lymphocytes through programmed cell death has also been demonstrated in animal models of lethal sepsis induced either by normal intestinal flora or by specific gram-negative bacteria. Studies using cecal ligation and perforation (CLP) in mice have shown profound lymphocyte apoptosis in multiple organs, including the thymus and spleen (*8*). Massive lymphoid apoptosis in the spleen and lymph nodes was also observed in baboons that developed fatal septic shock after injection of *Escherichia coli* (*9*).

Lymphocyte Apoptosis in Ebola Hemorrhagic Fever

In addition to occurring during common forms of sepsis, a marked increase in lymphocyte apoptosis has been observed in such exotic illnesses as Ebola hemorrhagic fever. When transferred to humans from an unidentified

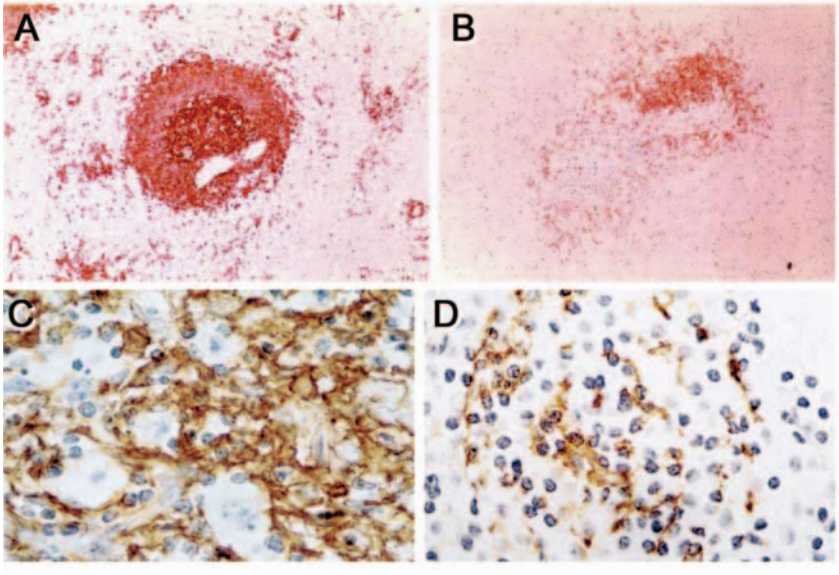

Figure 2. Immunohistochemical identification of B cells and follicular dendritic cells in spleens of patients dying of trauma or sepsis. Total B cells are decreased in the spleen of a patient with sepsis (B) compared with that of a trauma patient (A) (magnification ×400). Similarly, follicular dendritic cells are decreased in the spleen of a patient with sepsis (D) compared with that of a trauma patient (C) (magnification ×600).

animal reservoir, Ebola virus replicates rapidly in macrophages and dendritic cells, causing intense inflammation, high viremia, and spread of infection to multiple organs, with fever, coagulation abnormalities, and shock (*10*). Case fatality rates have reached 90% in outbreaks in central Africa.

Limited data from patients and more extensive data from laboratory animals indicate that massive lymphocyte apoptosis occurs during Ebola hemorrhagic fever and may contribute to the high death rate. Thus, the few patients who survive infection develop antibodies to the virus during the second week of illness, while fatally infected persons apparently undergo terminal immunosuppression similar to that seen with septic shock (*11*,*12*). A small study of blood samples from patients in Gabon showed that fatal cases of Ebola hemorrhagic fever were characterized by extensive intravascular apoptosis, particularly of T cells, beginning at least 5 days before death, with a decrease and eventual disappearance of Bcl-2 mRNA expression (*11*). In survivors, by contrast, Bcl-2 mRNA was identified in circulating cells during T-cell activation. Importantly, a similar loss of Bcl-2 has been reported in circulating lymphocytes of patients with sepsis (*4*).

Because of the difficulty of performing clinical research under the conditions of an Ebola outbreak, the pathogenesis of lethal infection has been elucidated principally through intensive studies in nonhuman primates, which develop uniformly lethal illness resembling fatal hemorrhagic fever in humans. Lymphocytes in these animals remain free of viral infection but nevertheless undergo extensive apoptosis, with early development of lymphopenia and depletion of circulating natural killer cells and CD4+ and CD8+ lymphocytes (*13*). Massive lymphocyte apoptosis is also observed histologically in lymph nodes, spleen, and other lymphoid tissues, beginning by day 3 postinfection. A model of Ebola virus infection in mice has demonstrated extensive lymphocytolysis in lymph nodes, spleen, and thymus, with histologic features suggestive of apoptosis (*14*). Lymphocyte apoptosis has also been demonstrated in vitro in cultures of Ebola virus–infected peripheral blood mononuclear cells, which suggests that infected monocytes release substances that induce apoptosis in neighboring lymphocytes (*15*).

Lymphocyte Apoptosis in Anthrax

In inhalational anthrax, spores of *Bacillus anthracis* are carried by pulmonary macrophages to mediastinal lymph nodes, where their replication results in local tissue injury, bacteremia, shock, and death (*16*). The ability of the organism to cause rapidly overwhelming infection suggests that, as in the case of Ebola hemorrhagic fever, immunosuppression plays a role in lethal illness. Few data are available from human cases to assess whether accelerated lymphocyte apoptosis contributes to this process, but a review of autopsy findings from 41 known cases of inhalational anthrax in a 1979 outbreak in Sverdlorsk, Russia, showed massive lymphocytolysis in mediastinal lymph nodes and spleens that was morphologically consistent with apoptosis (*17*).

Experimental evidence shows that lethal toxin (LT), an important virulence factor encoded by *B. anthracis*, interferes with intracellular signaling and can induce apoptosis. Ultrastructural analysis and terminal deoxynucleotidyl (TUNEL) staining of LT-treated human monocyte–derived dendritic cells found activation of apoptotic pathways (*18*). The same authors demonstrated that bone marrow dendritic cells from C57BL/6 and BALB/c mice differed in susceptibility to LT: cells derived from C57BL/6 mice underwent apoptosis and LT caused necrosis of equivalent cells from BALB/c mice.

Lymphocyte Apoptosis in Plague

The gram-negative bacillus *Yersinia pestis* causes 2 principal forms of illness in humans, a localized infection of lymph nodes (bubonic plague) and a highly lethal septicemia that is a particularly fulminant form of septic shock (*19*). The striking virulence of *Y. pestis* in humans is attributable to a collection of outer membrane proteins (Yops) that cause immune suppression and trigger apoptosis (*20*). Patients dying of plague would therefore be expected to demonstrate increased lymphocyte apoptosis, but data to support this hypothesis are lacking. However, laboratory studies using a murine model of intranasal *Y. pestis* infection have provided evidence of increased lymphocyte apoptosis in the spleen by 36 hours after infection (*21*) (R Hotchkiss, V. Miller, unpub. data).

YopH protein inhibits T cell activation by blocking early phosphorylation events necessary for signal transduction through the antigen receptor (*22*). In tests with primary T cells or Jurkat T leukemia cells, the extended presence of YopH led to apoptosis through a mitochondria-dependent pathway, as indicated by mitochondrial breakdown, caspase activation, DNA fragmentation, and annexin V binding. Cell death could be blocked through coexpression of Bcl-x_L, an antiapoptotic protein in the Bcl-2 family, or by treatment with caspase inhibitors. Evidence of induction of apoptosis was also found in a plague model in rats, in which increased numbers of caspase-positive cells were noted in lymph nodes 36 hours after infection, most prominently in nodes containing the greatest number of bacteria, which suggests Yop-mediated apoptosis (*23*). However, the apoptotic cells could not be identified because of extensive tissue destruction. Multifocal lymphocytolysis was also observed in the white pulp of the spleen, with resultant loss of periarteriolar lymphoid sheath–associated lymphocytes.

Experimental Inhibition of Apoptosis

Efforts to prevent excessive lymphocyte apoptosis during severe infection have focused either on modification of the signal processing system to create an inherent bias against the triggering of cell death pathways or on inhibition of caspase activity to block their execution. Proof-of-concept experiments with murine sepsis models have shown that both approaches can improve survival. Several studies have shown that transgenic mice overexpressing the antiapoptotic protein Bcl-2 were completely protected against lymphocyte apoptosis in T cells and partially protected in B cells after CLP and showed an increase in survival (*24*,*25*). The exact protective mechanisms, however, are unclear. The authors of 1 report argued that the beneficial effect of Bcl-2 did not depend on prevention of lymphocyte apoptosis because adoptive transfer of myeloid cells overexpressing Bcl-2 also resulted in improved survival after CLP of Rag-1–/– mice, which lack mature T and B cells (*25*). This finding suggests that protection resulted from the release of cytoprotective or antiinflammatory molecules from Bcl-2-overexpressing cells, from an increase in neutrophils at sites of infection, or both. Despite these findings, recent studies that showed a lower death rate after CLP in transgenic mice expressing the antiapoptotic protein Akt in T cells have added further support to the concept that prevention of lymphocyte apoptosis is an independent survival factor in sepsis (*26*).

In addition to these reports that used the CLP model, preliminary studies have shown that Bcl-2 overexpression prevents lymphocyte apoptosis in mice infected with *Y. pestis* (R. Hotchkiss, unpub. data). Bcl-2 transgenic mice that overexpressed Bcl-2 in T and B lymphocytes had a marked decrease in apoptosis at 72 hours after *Y. pestis* infection compared with wild-type animals (Figure 3). These findings provide hope that apoptotic cell death in plague may be preventable by a Bcl-2–based therapy.

Pharmacologic interventions have also been used to prevent initiation of lymphocyte apoptosis in murine models of sepsis (Table 2). One approach has aimed to block initial triggering of the extrinsic pathway by preventing cellular synthesis of Fas or FasL or by administering an inhibitor of Fas-FasL binding. Both techniques have shown benefit in murine CLP studies. Preliminary studies by Chung et al. demonstrated that mice genetically deficient in FasL showed better survival after CLP than their wild-type counterparts (*34*), and a survival benefit was also observed when mice were treated with siRNA to block intracellular synthesis of Fas (*28*). Markedly improved survival was also observed when a Fas receptor fusion protein was injected subcutaneously 12 hours after CLP to act as a decoy for FasL binding. Detailed studies have shown that this intervention restores normal immune function, improves cardiac output, and lowers the serum level of the antiinflammatory cytokine interleukin-10 (*27*).

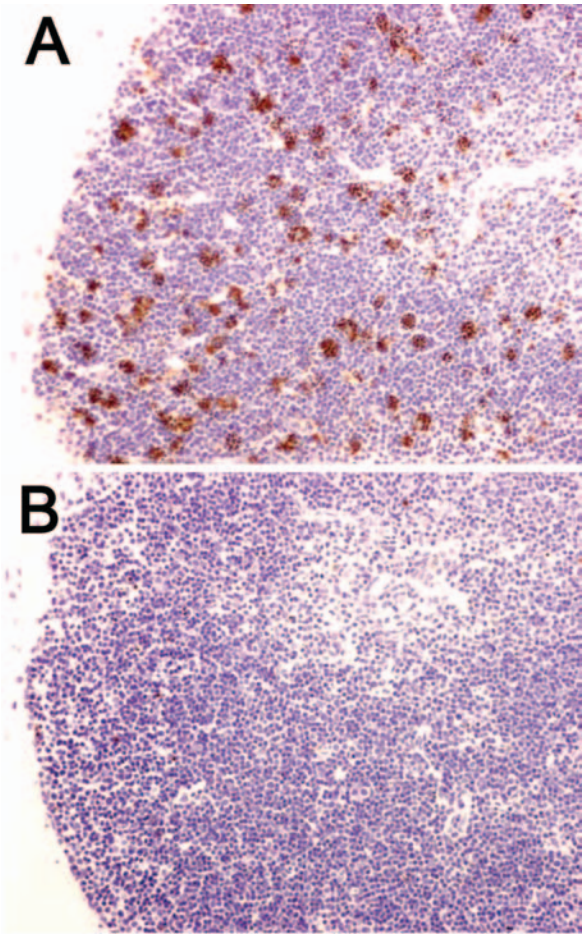

Figure 3. Decreased apoptosis caused by overexpression of Bcl-2 protein in a mouse model of plague. Wild-type mice (A) and mice that overexpressed Bcl-2 in lymphocytes (B) were injected intranasally with *Yersinia pestis*. Thymuses were obtained at 72 h postinfection and stained by using the terminal deoxynucleotidyl (TUNEL) method as a marker of apoptotic cell death. Note the decrease in apoptotic cells in the thymus of the Bcl-2 transgenic mouse (magnification ×400).

Another strategy aims to influence intracellular signaling networks in a direction opposing the initiation of programmed cell death. A recent publication by the Hotckhiss group showed that this could be achieved by exploiting the normal CD40 regulatory pathway through which lymphocytes are stimulated in an antiapoptotic direction to produce clonal expansion and functional maturation (*30*). Mice treated with a monoclonal antibody that binds to and stimulates the CD40 receptor showed up-regulation of the antiapoptotic protein Bcl-x_L, an absence of apoptosis of B cells, a decrease in loss of T cells, and a resistance to CLP (*29*).

Table 2. Therapeutic approaches for prevention of lymphocyte apoptosis in murine models of sepsis

Strategy	Intervention	Reference
Prevent triggering of extrinsic pathway	Blockade of Fas ligand by using Fas fusion protein	(*27*)
Prevent triggering of extrinsic pathway	Prevent Fas expression by using siRNA	(*28*)
Prevent initiation	Anti-CD40 agonist antibodies	(*29*)
Prevent initiation	Treatment with Bcl-2 agonist peptides	(*30*)
Prevent triggering of intrinsic pathway	Antiretroviral protease inhibitors	(*31*)
Prevent execution phase	Anticaspase-8 siRNA	(*28*)
Prevent execution phase	Treatment with caspase inhibitors	(*32*,*33*)

Efforts have also been made to alter intracellular signaling by introducing active portions of Bcl-x_L fused to carrier peptides to facilitate its transport into cells. In a murine CLP model, treatment resulted in a decrease in lymphocyte apoptosis, but the effect was less marked than that observed in transgenic animals constitutively expressing the same protein (*30*). Another approach has used the licensed HIV protease inhibitors nelfinavir and ritonavir, which in addition to blocking the cleavage of HIV propeptides have direct antiapoptotic effects (*31*). The latter were initially assumed to result from caspase inhibition, but further studies showed that these drugs prevent initiation of the intrinsic apoptotic pathway by stabilizing the mitochondrial membrane potential. Oral administration of nelfinavir and ritonavir to mice, beginning either before or 4 hours after CLP, resulted in decreased lymphocyte apoptosis and improved survival (*31*). Because both drugs are licensed for use in humans, this approach could potentially be evaluated in sepsis patients.

Efforts to prevent completion of the programmed cell death process by blocking executioner caspases have also been reported. Studies with the broad-spectrum caspase inhibitor zVAD showed decreased apoptosis and improved survival in a mouse CLP model (*32*). Similarly, a selective caspase-3 inhibitor decreased blood bacterial counts and improved survival in mice with sepsis (*33*). Treatment of septic Rag 1–/– mice with caspase inhibitors failed to improve survival, which suggests that the beneficial effect required the presence of lymphocytes.

Potential Limitations of Antiapoptotic Therapy

Although the proof-of-concept studies described above have shown promising results, deliberate inhibition of apoptosis during severe infections might have unexpected and undesired consequences. One potential adverse effect of antiapoptotic therapy involves its effects on pathogen replication. Some intracellular agents, such as poxviruses, actively inhibit apoptosis of their host cells so as to permit their own continued replication. Theoretically, pharmacologic inhibition of apoptosis in those situations could actually worsen the clinical outcome by providing an advantage to the pathogen. It may therefore be essential to identify the causative agent of infection before initiating antiapoptotic therapy. An alternative approach that may offer several advantages is targeted delivery of antiapoptotic molecules. Similar to current immune-based therapies, apoptosis inhibitors could be directed to specific classes of immune cells, for example by conjugating them to antibodies to CD4 or CD20, thus avoiding adverse consequences (*35*).

Other potential limitations of antiapoptotic therapy relate to possible undesired effects of the use of caspase inhibitors. First, because only a small amount of activated caspase-3 is sufficient to initiate genomic DNA breakdown and lead to apoptotic cell death, a high degree of inhibition would be needed to achieve therapeutic effectiveness (*36*). This requirement presents a therapeutic challenge because of the need for persistent and nearly complete caspase blockade. In addition, there is increasing recognition that caspases have numerous functions in addition to their roles as mediators of programmed cell death. One subset of caspases is critical for regulation of inflammation by processing proinflammatory cytokines such as interleukin-1β; others are essential for lymphocyte activation, proliferation, and protective immunity (*37*,*38*). Patients with defects in caspase-8, for example, are immunodeficient and have recurring infections (*39*). Blocking caspases might therefore have some beneficial effects in decreasing lymphocyte apoptosis in sepsis, but these could be counterbalanced by adverse effects on the ability of the patient to mount an effective immune response. Finally, that inhibition of caspases might induce hyperacute TNF-induced shock in certain situations has been recently reported (*40*). In view of the possible deleterious effects of using caspase inhibitors to treat sepsis, therapy directed at a temporary inhibition of specific caspases, such as caspase-3 or caspase-12, timed to either the hyperinflammatory phase or the hypoinflammatory phase of sepsis, might be the most effective approach.

Conclusions

A massive loss of lymphocytes and other cells through apoptosis is a proven component of the physiologic changes that occur over the course of septic shock. This process appears also to occur in a variety of other severe infections, including anthrax, plague, and Ebola hemorrhagic fever, which are of major concern for biodefense. A variety of proof-of-concept studies with murine sepsis

models have demonstrated that extensive apoptosis worsens disease outcome because its prevention through genetic modification or pharmacologic intervention improves survival. Research is needed to assess the possible contribution of lymphocyte apoptosis to immune impairment in other disease processes, including a variety of newly emerging infections. By helping to bolster immune function, the development of antiapoptotic therapies could mitigate the consequences of infection by a wide variety of pathogenic agents.

Dr Parrino is a clinical fellow in allergy and immunology at the National Institute of Allergy and Infectious Diseases. Her research interests include protocols focusing on new vaccines, emerging infectious diseases, and biodefense.

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Address for correspondence: Mike Bray, Biodefense Clinical Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 6700A Rockledge Dr, Rm 5128, Bethesda, MD 20892, USA; email: mbray@niaid.nih.gov

Reduced Efficacy of Insecticide-treated Nets and Indoor Residual Spraying for Malaria Control in Pyrethroid Resistance Area, Benin

Raphael N'Guessan,* Vincent Corbel,† Martin Akogbéto,‡§ and Mark Rowland¶

The pyrethroid knockdown resistance gene (*kdr*) has become widespread in *Anopheles gambiae* in West Africa. A trial to test the continuing efficacy of insecticide-treated nets (ITN) and indoor residual spraying (IRS) was undertaken in experimental huts at 2 sites in Benin, the first where *kdr* is present at high frequency (Ladji), the second where *An. gambiae* is susceptible (Malanville). Holes were made in the nets to mimic worn nets. At Malanville, 96% of susceptible *An. gambiae* were inhibited from blood-feeding, whereas at Ladji feeding was uninhibited by ITNs. The mortality rate of *An. gambiae* in ITN huts was 98% in Malanville but only 30% at Ladji. The efficacy of IRS was equally compromised. Mosquitoes at Ladji had higher oxidase and esterase activity than in a laboratory-susceptible strain, but this fact did not seem to contribute to resistance. Pyrethroid resistance in *An. gambiae* appears to threaten the future of ITN and IRS in Benin.

During the last decade, pyrethroid-treated mosquito nets have become the main method of malaria prevention in many malaria-endemic African countries (*1*,*2*). In a few notable exceptions, usually those with a more developed health infrastructure, such as South Africa, a long-standing practice of applying indoor residual spraying (IRS) has been successful (*3*). The 2 approaches to malaria prevention, insecticide-treated nets (ITNs) and spraying (IRS), are not mutually exclusive, and in malaria-endemic areas where ITN coverage is still limited, the feasibility of introducing IRS to reduce transmission is being considered, for example, by the President's Malaria Initiative Fund (*4*). Trials of IRS and ITNs have shown that in areas with pyrethroid-susceptible *Anopheles gambiae* the effectiveness of the 2 methods in controlling malaria does not differ (*5*). This comparability may not hold true for areas with pyrethroid-resistant populations. In southern Africa, for example, IRS with pyrethroid failed to control pyrethroid-resistant *An. funestus* and necessitated a switch to an alternative class of insecticide to which there was no resistance (*6*). During the last decade, pyrethroid resistance caused by the *kdr* mechanism has become widespread in *An. gambiae* in West Africa and is common in some areas (*7*). Whether *kdr* undermines the effectiveness of ITN in areas of high prevalence is unclear. An early experimental hut trial of ITNs in Côte d'Ivoire demonstrated a survival advantage of homozygotes for *kdr* resistance (*8*), whereas subsequent hut trials in adjacent resistant and susceptible populations showed no apparent difference in the effectiveness of ITNs between the 2 localities (*9*). Village randomized trials in Côte d'Ivoire showed that ITNs continued to prevent malaria despite a vector population that was *kdr* resistant (*10*). Whether *kdr* would undermine the effectiveness of IRS in the same way as resistance due to oxidases did against *An. funestus* in southern Africa (*6*) is unknown. To assess the practicability of applying IRS with pyrethroid in West Africa, we need to examine the effectiveness of this approach against a *kdr*-resistant population of *An. gambiae*. To get a clearer understanding of the influence of *kdr* resistance on the effectiveness of ITN, further

*London School of Hygiene and Tropical Medicine, Cotonou, Benin, West Africa; †Institut de Recherche pour le Developpement, Montpellier, France; ‡University of Abomey-Calavi, Cotonou, Benin; §Centre de Recherche Entomologique, Cotonou, Benin; and ¶London School of Hygiene and Tropical Medicine, London, United Kingdom

experimental hut trials of ITNs against *kdr*-resistant populations need to be conducted. We describe 2 experimental hut trials in Benin. One compares the impact of IRS and ITN against a pyrethroid- resistant population in the southern part of the country; the other compares IRS and ITNs against a pyrethroid-susceptible population several hundred kilometers to the north.

Material and Methods

Study Sites

Ladji is a large village on the outskirts of Cotonou, the capital of Benin. The village floods during the rainy season. *An. gambiae* comprises the Mopti (M) cytotype and shows resistance to pyrethroids and DDT; *kdr* is present at high frequency (*11*). The nuisance mosquito *Culex quinquefasciatus* is also present and shows resistance to pyrethroids. Five experimental huts belonging to the Centre de Recherche Entomologique de Cotonou (CREC) are situated in the village.

Malanville is in northern Benin, 800 km from Cotonou, in an irrigated rice-growing valley. The local *An. gambiae* comprises the M cytotype, but the *kdr* gene is almost absent and mosquitoes are susceptible to lambdacyalothrin and deltamethrin. Six experimental huts are present at Malanville.

Experimental Huts

The treated nets, residual spray treatments, and their respective untreated controls were evaluated in 4 experimental huts at each field site. Experimental huts are specially designed to test vector control products against freely entering mosquitoes under natural but controlled conditions. Huts were typical of the region. Each was made from concrete bricks, with a corrugated iron roof and a ceiling of thick polyethylene sheeting lined with hessian sackcloth on the interior surface, and each was built on a concrete base surrounded by a water-filled moat to exclude ants (*12*). Mosquito access was through 4 window slits, constructed from pieces of plywood fixed at an angle to create a funnel with a 1-cm gap, present on 3 sides of the huts. Mosquitoes had to fly upward to enter through the gaps and downwards to exit; this precluded or limited exodus through the aperture and enabled us to account for most entering mosquitoes. A veranda trap projected from the back wall of each hut. Movement of mosquitoes between a room and the veranda was unimpeded.

Mosquito Net Treatments

The nets were made of white, 100-denier polyester (SiamDutch Mosquito Netting Co., Bangkok, Thailand). Nets measured 2.0-m long, 1.6-m wide, and 1.8-m tall and had a surface area of 16.9 m^2. To simulate badly torn nets, 80 holes, each measuring 2 × 2 cm, were cut in the sides and ends of each net.

Insecticides used were formulations of lambdacyhalothrin (Icon, Syngenta, Switzerland): lambdacyhalothrin 2.5% CS, a microencapsulated suspension designed for ITNs, and lambdacyhalothrin 10% WP, a wettable powder designed for IRS.

The lambdacyhalothin application rates of 18 mg/m^2 for ITNs and 30 mg/m^2 for IRS were within the ranges recommended by the manufacturer. Indoor residual treatments were applied with a hand-operated compression sprayer equipped with a flat fan nozzle. The cement walls and sackcloth ceilings were sprayed uniformly after masking the veranda and window slits with protective coverings. The control hut was sprayed with water only. The treated huts were left for 1 week before evaluations were started.

Sleepers and Mosquito Collections

Preliminary experiments showed the huts to be evenly attractive to mosquitoes. The treatments were randomly allocated to the 4 experimental huts at each site. The main trials were conducted from April to June 2005 at the Ladji site and from September to November 2005 at the Malanville site. Eight adult men employed by CREC slept overnight in the huts and collected mosquitoes from the huts in the mornings. Informed consent to participate in the study was given beforehand, and chemoprophylaxis was provided during the trial. Ethical approval was granted by the London School of Hygiene and Tropical Medicine (LSHTM) and Benin national ethics committees.

The trial ran for 50 nights for 8 weeks at each site. The sleepers were rotated between huts to correct for possible variation in individual attractiveness. Each morning, mosquitoes were collected from the floors, walls, and ceilings of rooms, verandas, and nets with aspirators and torches. Mosquitoes were identified and scored as blood-fed or unfed and dead or live. Live mosquitoes were held in netted plastic cups and supplied with 10% honey solution for 24 h before delayed mortality was recorded. Male mosquitoes were not scored.

The entomologic impact of each treatment on mosquitoes was expressed relative to the control in terms of the following: deterrence, the proportional reduction in the number of mosquitoes entering a treated hut relative to that entering the control hut; induced exophily, the proportion of mosquitoes collected from the veranda trap of the treatment hut relative to the proportion in the veranda of the control hut; blood-feeding inhibition, the reduction in blood-feeding rate relative to the control hut; and mortality, the proportions of mosquitoes found dead in the hut at the time of collection and after a 24-h holding period.

If a treatment deters a considerable number of mosquitoes from entering the hut, the values given by proportion blood-feeding or proportion killed in the treatment hut may underestimate the full personal protective effect and overestimate the full insecticidal efficacy of the treatment. The personal protective effect of a treatment is better described by the reduction in the number of blood-fed mosquitoes in the treatment hut relative to the number blood-fed in the control hut:

$$\% \text{ Personal protection} = 100\ (B_u - B_t)/B_u$$

where B_u is the total number of blood-fed mosquitoes in the untreated control huts and B_t is the total number blood-fed mosquitoes in the huts with insecticide treatment.

The overall insecticidal effect of a treatment needs to take into account that a considerable number of mosquitoes might be deterred from entering the hut and hence not be killed by the treatment. A mass killing effect is desirable to reduce transmission. The overall insecticidal effect of a treatment relative to the number of mosquitoes that would ordinarily enter an untreated hut can be estimated by using the following formula and expressed as a percentage:

$$\text{Overall insecticidal effect } (\%) = 100\ (K_t - K_u)/(T_u - K_u)$$

where K_t is the number killed in the treated hut, K_u is the number dying in the untreated control hut, and T_u is the total number collected from the control hut.

Residual Activity of Insecticide Treatments

To evaluate residual activity, World Health Organization (WHO) cone bioassays were undertaken monthly in the Ladji huts and bimonthly in the Malanville huts with a laboratory-susceptible strain of *An. gambiae* (Kisumu). *An. gambiae* females, 3–5 days old, were exposed within the cones to nets for 3 min or to sprayed walls and ceilings for 30 min. Approximately 50 mosquitoes in 5 replicates of 10 mosquitoes were tested on each substrate. Honey solution was provided during the 24-h holding period, and the temperature was kept at 25°C.

Biochemical Assays

Biochemical tests on individual mosquitoes were conducted to determine the activity of mixed function oxidases and nonspecific esterases present in pyrethroid-resistant and -susceptible samples of *An. gambiae* from the Ladji and Malanville sites. Tests were conducted on 3-day-old adult females (initially collected as larvae) in microtiter plates (*13*). Susceptible (Kisumu) and pyrethroid-resistant (Vkper) *An. gambiae* served as controls. Genotyping of *An. gambiae* was carried out to assess *kdr* frequency at both field stations (*14*).

Adult Bioassay Data

To determine whether a stronger pyrethroid resistance mechanism was present in the Ladji population than in the standard *kdr* strain Vkper, bioassays with 0.05% lambdacyalothrin-treated papers (18 mg/m^2) were conducted in WHO resistance test kits by using a range of exposure times on batches of 25 unfed *An. gambiae* females 2–5 days of age. One hundred mosquitoes per exposure period were tested. Deaths were scored 24 h later. Log-time mortality curves were generated, and lethal time to kill 50% (LT_{50}), estimated by using probit analysis.

Data Analysis

Proportional data from the hut trial (exophily, blood-feeding, deaths) were analyzed by using logistic regression (STATA 6 software**,** Stata Corporation, College Station, TX, USA). Deterrence rates were analyzed by comparing the number of mosquitoes entering each hut by using the Wilcoxon rank sum test. Biochemical activity was analyzed with Kruskal-Wallis and Wilcoxon rank sum tests. The level of resistance to lambdacyalothrin in insecticide bioassays was analyzed by using probit analysis.

Results

Insecticide Residual Activity

Residual activity on ITN as measured by cone bioassay tests showed no decline during the 8 weeks of the trial. Activity of the IRS wettable powder formulation on sackcloth and cement showed a decline in performance by week 4. This trend continued until the end of the trial (Table 1).

Efficacy of Treatments in Huts

Over the 2-month trial, 1,395 *An. gambiae*, 3,070 *Cx. quinquefasciatus*, and small numbers of *Mansonia uniformis*, *An. pharoensis*, and *Aedes aegypti* were collected at Ladji. At Malanvile, 1,523 *An. gambiae*, 2,804 *Mansonia* sp., and smaller numbers of *An. funestus* and *Ae. aegypti* were collected. Only the malaria vector *An. gambiae* and the nuisance mosquito *Cx. quinquefasciatus* were analyzed further.

Fewer *An. gambiae* entered the ITN- and IRS-treated huts than the respective control huts. The treatment induced reduction in hut entry was more evident in the resistance area than in the susceptible area (Table 2). The proportion deterred at each site did not differ between ITN or IRS treatments.

The untreated net was little or no barrier to blood-feeding of *An. gambiae* at either field site owing to the large number of holes cut in each net. Treating the holed net with pyrethroid led to a 96% reduction in the number of mosquitoes blood-feeding at the susceptible site

Table 1. Residual activity of lambdacyalothrin (insecticide)-treated nets (ITNs) and indoor residual spraying over 3 mo in experimental huts, Malanville and Ladji field stations*

When and where substrate tested	ITNs at 18 mg/m^2		Indoor residual spraying at 30 mg/m^2			
	Sides + top of net		Ceiling		Walls	
	No. tested	% Corrected mortality	No tested	% Corrected mortality	No. tested	% Corrected mortality
Wk 0						
Malanville	77	100	33	100	60	100
Ladji	51	100	30	93.3	54	100
Wk 2						
Ladji	52	100	22	100	41	100
Wk 4						
Ladji	54	100	21	52.4	47	42.5
Wk 6						
Ladji	57	100	25	80.0	45	31.1
Wk 8						
Malanville	52	100	29	41.4	54	2.6
Ladji	44	97.7	8	25.0	39	18.5

*As determined by using World Health Organization cone bioassays and susceptible *Anopheles gambiae* (Kisumu).

(Malanville) but to no reduction in blood-feeding at the resistant site (Ladji). Inhibition of blood-feeding by IRS at either the resistant or susceptible site was limited (Table 2).

Natural mortality of *An. gambiae* occurred in both types of control huts but was notably higher at Ladji than at Malanville. Both modes of treatment were highly insecticidal at Malanville: ITNs treated with 18 mg/m^2 lambdacyhalothrin killed 99%, and IRS applied at 30 mg/m^2 killed 72% of *An. gambiae* that entered the huts. At Ladji, the proportions of *An. gambiae* killed in either the ITN- or IRS-treated hut did not exceed 30% (Table 2).

The proportion of *An. gambiae* collected from the veranda traps in the mornings was greater at Malanville than at Ladji and greater in the huts with untreated nets than in the unsprayed control huts. Relative to the controls, lambdacyalothrin-treated nets and IRS induced little or no exophily of the pyrethroid-resistant *An. gambiae* into the verandas of the Ladji huts, despite high survival rate of mosquitoes in huts. At Malanville, pyrethroid-induced exophily by ITN or IRS hut was not evident and may have been obscured by the high death rates among the mosquitoes.

Table 2. Experimental hut results of lambdacyhalothrin (insecticide)-treated nets (ITNs) and indoor residual spraying (IRS) against *Anopheles gambiae*, Ladji (pyrethroid resistance) and Malanville (pyrethroid susceptibility) field stations*

ITNs	Ladji (pyrethroid resistance)		Malanville (pyrethroid susceptibility)	
	Untreated net	Lambdacyhalothrin 18 mg/m^2	Untreated net	Lambdacyhalothrin 18 mg/m^2
Total collected	689†	386‡	363†	267‡
Deterred, %	–	44.0	–	26.4
Exiting, % (CI)	25.0 (21.7–28.2)†	29.0 (24.5–33.5)†	36.1 (31.1–41.0)†	46.8 (40.8–52.8)‡
Blood-fed, % (CI)	82.0 (79.1–84.9)†	82.1 (78.3–85.9)†	77.7* (73.4–81.9)†	3.0* (0.9–5.0)‡
Blood-feeding inhibition, %	–	0	–	96.1
Personal protection, % (no. blood-fed)	– (572)†	44.6 (317)‡	– (282)†	97.2 (8)‡
% Dead (CI)	13.6* (11.1–16.2)†	29.8* 25.2–34.4)‡	3.6* (1.7–5.5)†	98.5* (97.0–99.9)‡
Insecticidal effect, % (no. dead)	– (94)†	3.0 (115)†	– (13)†	68.9 (263)‡
IRS	**Unsprayed hut**	**Lambdacyhalothrin 30 mg/m^2**	**Unsprayed hut**	**Lambdacyhalothrin 30 mg/m^2**
Total collected	203†	117‡	498†	395‡
Deterred, %	–	42.4	–	20.7
Exiting, % (CI)	45.8 (38.9–52.7)†	58.1 (49.2–67.1)†	54.4 (50.0–58.8)†	63.3 (58.5–68.0)†
Blood-fed, % (CI)	87.7* (83.2–92.2)†	73.5* (65.5–81.5)†	93.8* (91.6–95.9)†	69.6* (65.1–74.2)‡
Blood-feeding inhibition, %	–	16.2	–	25.8
Personal protection, % (no. blood-fed)	– (178)†	51.7 (86)‡	– (467)†	41.1 (275)‡
Dead, % (CI)	12.3* (7.8–16.8)†	30.8* (22.4–39.1)‡	1.4* (0.4–2.4)†	72.1* (67.7–76.6)‡
Insecticidal effect, % (no. dead)	– (25)†	5.4 (36)†	– (7)†	55.8 (285)‡

*For each untreated–treated pair, values not sharing the same symbols († or ‡) are significantly different at the 5% level. CI, 95% confidence interval.

The personal protection derived from ITN was almost 100% in the susceptible area. Despite the low mortality rate and high rate of blood-feeding observed with ITN in the resistance area, the level of personal protection there was almost 50% because of the deterrent effect of lambdacyhalothin on mosquito entry into huts. The personal protective effect of IRS was low in both areas, and IRS was no barrier to blood-feeding. The overall insecticidal effect of pyrethroid-treated nets and IRS was negligible in the resistance area (≤5.4%) but was considerable in the susceptible area (≥55.8%).

Table 3 breaks down the mortality data into 2-week blocks. Mortality associated with IRS treatments decreased week by week at both sites but started at a lower rate at the Ladji site because of the expression of resistance. Mortality associated with ITN treatments also showed a downward trend over time at Ladji but not at Malanville, where mosquitoes showed high susceptibility throughout the study.

Both ITN and IRS treatments at Ladji showed poor efficacy against *Cx. quinquefasciatus* (this species was not encountered in Malanville). Insecticide-induced deterrence was greater for ITN than for IRS (Table 4). Neither method killed many *Culex* nor stimulated repellency into verandas. The IRS treatment produced an unusually high level of blood-feeding inhibition.

Biochemical Assays and *kdr* Genotyping

An. gambiae from Ladji expressed a significantly higher level of oxidase activity than the standard susceptible (Kisumu) and the laboratory *kdr* (Vkper) strains, which had a similar level of oxidase activity. However, the pyrethroid-susceptible strain from Malanville showed a level of oxidase activity that was not significantly different from that of the Ladji strain. This finding would appear to rule out any contribution from oxidases to the pyrethroid resistance observed in *An. gambiae* from Ladji. The level of α-esterase activity in *An. gambiae* from Ladji was significantly higher than that expressed in Malanville or Kisumu strains, whereas the level of β-esterase activity in Ladji, Vkper, and Kisumu strains was similar and clearly played no part in resistance (Table 5). Overall, the mean level of esterase activity at Malanville was significantly lower than that of the susceptible reference strain ($p<0.05$). Genotyping data (Table 6) showed a high frequency of *kdr* resistance at Ladji (F [*kdr*] = 83%, n = 45) and low frequency at Malanville (F [*kdr*] = 6%, n = 45). The pyrethroid- resistant Vkper was fixed for the *Kdr* gene (F [*kdr*] = 100%, n = 47).

Adult Bioassays

The summary results of the exposure time mortality bioassays with lambdacyhalothin-treated papers in WHO cylinder kits are shown in Table 6. The slopes and LT_{50}s of the probit regression curves were not significantly different for Ladji and Vkper strains. Tests on the Kisumu strain produced 100% mortality after only 1 min exposure. An LT_{50} could not be calculated by using probit analysis, but the resistance factor in the Ladji and Vkper strains was at least 10-fold.

Discussion

A major loss of efficacy associated with pyrethroid resistance occurred in *An. gambiae* at Ladji, Benin. The reduction in efficacy affected IRS and ITNs equally: only 19% of mosquitoes in the ITN hut and only 22% in the IRS hut were killed after correction for natural mortality. By contrast, 98% of mosquitoes entering the ITN hut and 72% entering the IRS hut located in the susceptible north of Benin were killed by the lambdacyhalothin treatments after correction for natural mortality. These findings are the first clear evidence of pyrethroids' failing to control an *An. gambiae* population that contains *kdr* resistance at high levels. Whereas the loss of insecticidal effect was calculated to be ≥95%, a degree of personal protection associated with ITNs and IRS was still evident (45%–50%) relative to the untreated net or unsprayed hut owing to a partial deterrent effect of treatments on entry of mosquitoes rather than to any inhibition of blood-feeding once the insects were inside the huts. Indeed, on entering the huts, most mosquitoes did go on to blood-feed, and the deliberately holed ITN was no barrier to resistant mosquitoes. By contrast, in northern Benin, only 4% of the insecticide-susceptible mosquitoes that entered the hut fed through the holed ITN. The loss of personal protection and loss of mosquito mortality associated with resistance would presumably com-

Table 3. Mortality rate of free-flying, naturally entering mosquitoes in huts, first 8 weeks of trial*

	Ladji (pyrethroid-resistant *Anopheles gambiae*)				Malanville (pyrethroid-susceptible *An. gambiae*)			
	ITN		IRS		ITN		IRS	
Wk	No.	% Corrected mortality	No.	% Corrected mortality	No.	% Corrected mortality	No.	% Corrected mortality
1–2	41	43.2	15	53.3	67	100	91	100
3–4	83	50.5	42	47.6	93	100	108	88.7
5–6	209	28.7	39	24.2	54	92.6	78	57.8
7–8	53	5.7	21	23.8	53	98.8	118	39.0

*ITN, insecticide-treated net; IRS, indoor residual spraying.

Table 4. Experimental hut results of lambdacyalothrin (insecticide)-treated nets (ITNs) and indoor residual spraying (IRS) against *Culex quinquefasciatus*, Ladji (pyrethroid resistance) field station*

	Treatments			
	ITN		IRS	
Results	Untreated net	Lambdacyhalothrin 18 mg/m^2	Unsprayed hut	Lambdacyhalothrin 30 mg/m^2
Total entered	845†	598‡	858†	769†
Deterred, %	–	29.2	–	10.4
Exiting, % (CI)	29.8 (26.7–32.9)†	35.9 (32.1–39.8)‡	52.7 (49.3–56.0)†	54.6 (51.1–58.1)†
Blood-fed, % (CI)	62.8 (59.6–56.1)†	59.5 (55.6–63.5)†	85.1 (82.7–87.5)†	42.9 (39.4–46.4)‡
Blood-feeding inhibition	–	NS	–	49.6
Personal protection, % (no. blood-fed	– (531)†	33.1 (355)‡	– (730)†	54.8 (330)‡
Dead, % (CI)	4.3 (2.9–5.6)†	8.5 (6.3–10.8)‡	3.4 (2.2–4.6)†	16.3 (13.7–18.9)‡
Insecticidal effect, % (no. dead)	– (36)†	1.9 (51)†	– (29)†	11.6 (125)‡

*For each untreated–treated pair, values not sharing the same symbols († or ‡) are significantly different at the 5% level. CI, 95% confidence interval; NS, not significant.

bine to make ITNs unattractive from the perspective of both the individual user and the malaria control manager. Incision of 80 holes per net is the standard for ITN trials in West Africa (*8*,*9*,*12*), and such nets have given a degree of personal protection in earlier trials. An ITN with no or few holes might be expected to give some protection against resistant mosquitoes from Ladji, but there were insufficient huts available to test this idea.

These experimental hut results from southern Benin stand in contrast to results from an area of Côte d'Ivoire (Yaokoffikro) that had a comparable frequency of *kdr* (78%) to that of Ladji (83%) (*15*) and where lambdacyhalothrin-treated nets and other ITN showed continuing efficacy, with mortality rates of 45%–68% (*8*,*16*–*19*).

We sought evidence that other resistance mechanisms than *kdr* might be contributing to the reduced efficacy of pyrethroids at Ladji. Metabolic resistance due to mixed function oxidases (MFO) has, for example, undermined attempts at malaria control with deltamethrin residual spraying in southern Africa caused by *An. funestus* (*6*), and elevated MFO activity in a strain of *An. gambiae* from Cameroon reduced the efficacy of permethrin-treated netting in laboratory tests (*20*). The combined elevated activity of MFOs, glutathione S–transferase, and esterases resulted in a failure of the S. Mexican IRS program against *An. albimanus* (*21*). Our examination of enzymatic activity in *An. gambiae* showed no evidence that MFO activity is any greater in mosquitoes from Ladji than in mosquitoes from Malanville, nor did esterase activity differ between Ladji and Vkper (*kdr*) strains. Thus, there was no evidence of metabolic resistance enhancing the resistance already caused by *kdr* in mosquitoes from Ladji. Lambdacyhalothin bioassay tests showed no evidence of resistance level differing between Ladji and Vkper strains, and we conclude that metabolic mechanisms made no contribution to the observations in Ladji.

Table 5. Efficacy of lambdacyalothrin-treated filter papers* to *Anopheles gambiae* from Ladji, Vkper (fixed for *kdr* allele) and Kisumu (susceptible) strains†

	Filter paper bioassays treated with lambdacyalothrin 0.05% (18 mg/m^2)		
Strains	Slope (SE)	LT_{50} (95% CI)	LT_{50} ratio
Ladji	2.1 (0.2)	10.9 (7.2–14.8)	
Vkper	2.1 (0.2)	14.2 (3.6–25.3)	1.3 (1.0–1.6)
Kisumu		<1	

*In World Health Organization kits.
†As determined by using probit analysis. *kdr*, knockdown resistance; SE, standard error; CI, confidence interval; LT_{50} is the exposure time in minutes to kill 50%.

In East Africa a different type of *kdr* based on a leucine-to-serine mutation, which confers resistance to permethrin and DDT (*22*), has been detected in several countries. However, no mosquitoes of this genotype were detected in tests on samples of *An. gambiae* from Ladji (*23*). The complete absence of efficacy of lambdacyalothrin against *Cx. quinquefasciatus* in Ladji merely confirms earlier findings involving other types of pyrethroid in experimental huts in West Africa (*6*,*9*,*16*,*18*).

The contribution of *kdr* to pyrethroid resistance in *An. gambiae* needs to be reappraised. While lambdacyhalothin-treated nets (reported here) and permethrin-treated nets reported earlier (*24*) were less effective in hut trials in the *kdr* area of Benin (Ladji) than in a corresponding area of Côte d'Ivoire (Yaokoffikro), pyrethroid-treated nets were more effective in the susceptible area of Benin (Malanville) than in the corresponding susceptible area of Côte d'Ivoire (M'Be) (*9*) for reasons that are presently unknown. Other differences between the biology of *An. gambiae* from Côte d'Ivoire and Benin exist. Ivorian *An. gambiae* with *kdr* is mainly of the S molecular form, whereas Beninoise *An. gambiae* is of the M form (V. Corbel, unpub. data). M and S forms differ in ecologic distribution and habitat. While mosquitoes of the M form with *kdr* might behave differently from those of the S form with *kdr* when exposed to pyrethroids, this is mere speculation. Moreover, the M form in Malanville showed higher vul-

Table 6. Molecular and biochemical assays* conducted on samples of *Anopheles gambiae* from Malanville and Ladji compared with laboratory-susceptible (Kisumu) and pyrethroid-resistant *kdr* (Vkper) strains†

Populations or strains	N	Frequency of *kdr* (%)	Oxidase nmol P450 U/mg†	α-esterase μmol/min/mg	β-esterase μmol/min/mg
Kisumu	40	0	0.15 (± 0.020)‡	0.11 (± 0.019)‡§	0.12 (± 0.016)‡
Malanville	45	6	0.25 (± 0.018)§	0.07 (± 0.017)‡	0.04 (± 0.015)§
Ladji	45	83	0.27 (± 0.018)§	0.18 (± 0.017)¶	0.15 (± 0.014)‡¶
Vkper	47	100	0.13 (± 0.017)‡	0.11 (± 0.017)§¶	0.14 (± 0.014)¶

*Mean enzymatic activity ± SE (standard error).
†In each column, values not sharing the same symbols (‡, §, or ¶) are significantly different at the 5% level.

nerability to ITN than did the corresponding S form in Côte d'Ivoire, a finding that seems at odds with a behavioral hypothesis.

Our study provides persuasive evidence that pyrethroid resistance in Benin is capable of undermining control measures based on ITN. Nor is there reassurance to be taken from IRS, and any attempt to switch vector control strategy would seem doomed to fail. Whereas the earlier phase 3 malaria control trials of ITN in Côte d'Ivoire showed continuing effectiveness despite *kdr* at high levels (*10*), our phase 2 results from Benin give no grounds for optimism. However, only phase 3 can provide a definitive answer. Further phase 3 trials using pyrethroid-treated nets and IRS need to be undertaken in Benin in an area of pyrethroid resistance. The normal practice with phase 3 is to aim at complete community coverage. Coverage in real life is usually less than total, and the danger with the type of pyrethroid resistance found in Benin is that at lower levels of coverage the important mass protective effect of ITNs (*25*,*26*) may be lost and transmission may continue unabated among those who do not have ITNs. To establish whether this is true, phase 3 trials on resistant mosquito populations should ideally set the coverage level at <100%. If it is considered unacceptable to deny a section of the trial population access to ITNs, an alternative but much less rigorous approach would be to monitor malaria incidence among users and nonusers of long-lasting insecticide nets (LLIN) during the proposed scaling up of LLIN coverage in Benin currently being considered.

Pyrethroid resistance in Benin is far from homogeneous, and LLIN should give good protection wherever mosquito populations are susceptible. Use of LLIN should be encouraged but scale-up of treated nets may ultimately select for further resistance. The need to develop alternative insecticides to replace or supplement pyrethroids on nets is urgent and should be put on a par with the seeking of new antimalarial drugs or vaccines that have received far greater attention and resources in recent years.

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Mr N'Guessan of LSHTM is the Gates Malaria Partnership Project manager based in Benin. His current research interests are on insecticide resistance in *Anopheles gambiae* and identifying alternative insecticides to maintain the effectiveness of insecticide-treated materials in West Africa.

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Address for correspondence: Mark Rowland, London School of Hygiene and Tropical Medicine, Keppel St, London WC1E 7HT, United Kingdom; email: mark.rowland@lshtm.ac.uk

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Code-based Syndromic Surveillance for Influenzalike Illness by International Classification of Diseases, Ninth Revision

Nicola Marsden-Haug,*[1] Virginia B. Foster,* Philip L. Gould,† Eugene Elbert*[2] Hailiang Wang,* and Julie A. Pavlin*[3]

With the spread of avian influenza, use of automated data streams to rapidly detect and track human influenza cases has increased. We performed correlation analyses to determine whether International Classification of Diseases, Ninth Revision (ICD-9), groupings used to detect influenzalike illness (ILI) within an automated syndromic system correlate with respiratory virus laboratory test results in the same population (r = 0.71 or 0.86, depending on group). We used temporal and signal-to-noise analysis to identify 2 subsets of ICD-9 codes that most accurately represent ILI trends, compared nationwide sentinel ILI surveillance data from the Centers for Disease Control and Prevention with the automated data (r = 0.97), and found the most sensitive set of ICD-9 codes for respiratory illness surveillance. Our results demonstrate a method for selecting the best group of ICD-9 codes to assist system developers and health officials who are interpreting similar data for daily public health activities.

Inevitable annual cycles of influenza and other respiratory pathogens pose a significant threat to work and productivity (*1*–*3*). Epidemics can have dramatic economic and medical ramifications, such as the influenza pandemic of 1918 (*4*,*5*). During the last few years we have witnessed the emergence of severe acute respiratory syndrome (SARS) and new pathogenic avian influenza strains. These events have brought respiratory illnesses to the attention of the general public; most recently, the highly publicized potential for pandemic influenza due to recombinant influenza strains has generated tremendous public anxiety. Moreover, lingering fears about influenzalike illness (ILI) symptoms related to bioterrorism have further accentuated the need for improved early detection of respiratory disease outbreaks.

This atmosphere of concern motivated an intense effort to develop new surveillance methods (*6*). Public health officials are now augmenting traditional disease surveillance, e.g., laboratory-based methods, with nontraditional analysis of electronic medical records for more timely monitoring of infectious disease patterns. The Centers for Disease Control and Prevention (CDC), along with many health departments, universities, and government organizations, has participated in research and development of syndromic surveillance systems. Some of these systems have been designed for local surveillance in a single metropolitan area, while others cover broad geographic areas, including multiple jurisdictions (*7*,*8*).

Since 2001, the Department of Defense (DOD) has been using the Electronic Surveillance System for the Early Notification of Community-based Epidemics (ESSENCE) for syndromic surveillance of active duty military and their beneficiaries (*9*,*10*). This system captures patient ambulatory data coded according to the International Classification of Diseases, Ninth Revision (ICD-9), from all permanent military treatment facilities

*Walter Reed Army Institute of Research, Silver Spring, Maryland, USA; and †Air Force Institute for Operational Health, Brooks City Base, Texas, USA

[1]Current affiliation: Tacoma–Pierce County Health Department, Tacoma, Washington, USA

[2]Current affiliation: US Census Bureau, Suitland, Maryland, USA

[3]Current affiliation: Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

(MTFs) that treat active duty personnel, retirees, and their family members worldwide. It provides a large amount of data for surveillance, with >300,000 average weekly outpatient visits to primary care and emergency facilities for any reason. The system automatically performs daily analysis of visits classified in each of 8 syndrome groups, such as respiratory, gastrointestinal, and febrile illnesses.

Military basic training sites have historically experienced frequent respiratory epidemics among troops in crowded housing (*11–14*), and active surveillance for ILI is conducted year-round. To improve early detection of such epidemics and in response to pandemic and bioterrorism concerns, an automated ILI surveillance report was also incorporated into ESSENCE in 2002 (*9*).

Critics of syndromic surveillance have voiced apprehension about the use of nontraditional data and the ability of these systems to detect outbreaks (*15–17*). Skepticism about ICD-9 data in particular revolves around whether data coded at the time of visit accurately reflects true illness, given the potential for coding of nonspecific symptoms and unconfirmed diagnoses and for provider or coder variations in code selection (*18*). We sought to evaluate the effectiveness of using ESSENCE as an early detection system for ILI and to determine the most parsimonious set of ICD-9 codes to use for ILI surveillance. We compared the ICD-9–based ILI data in ESSENCE to data from the laboratory-based DOD Global Influenza Surveillance Program and the sentinel reports from CDC's US Influenza Sentinel Providers Surveillance Network. We compared diagnostic codes from ESSENCE both individually and as a group to the volume of positive respiratory specimens and weekly sentinel reports. Through trend, correlation, and signal-to-noise analysis, we identified a subset of diagnostic codes that best corresponds with influenza patterns.

Methods

ESSENCE Data Collection

ESSENCE captures outpatient visit data recorded as ICD-9 codes at or shortly after the patient encounter (*10*). A central, secure-link electronic database allows for daily submission of data, although reporting time from the MTFs averages from 1 to 4 days. Data entry practices vary by location, but each MTF is set up to batch-send data to the central database on a daily basis; in most locations, 80% of all ICD-9 codes are received within 4 days. The ESSENCE server collects de-identified data from the central database every 8 hours; at each time of collection, ESSENCE is refreshed with newly submitted data from MTFs. With each cycle, data are grouped by ICD-9 codes, recounted, and republished into syndromes, including ILI. Most syndromes are published as daily counts, but the ILI syndrome is grouped as weekly data. The published data for the ILI syndrome is also updated and republished every 8 hours, but the initial publication of the weekly data does not occur until a full week (running Sunday to Saturday) is completed.

We created our original ILI syndrome group by reviewing the ICD-9 code and listing and choosing those that could represent potential ILI cases. According to this classification, visits are counted as ILI if their diagnostic code is either fever, an included acute respiratory code, or unspecified viral illness. The 29 codes in the original ILI group are listed in Table 1. Each week ESSENCE calculates the percentage of visits for ILI among the total number of outpatient primary care and emergency department visits.

Direct Comparison of Respiratory Specimens Matched to Outpatient Visits

The DOD Influenza Surveillance program, located at the Air Force Institute for Operational Health at Brooks Air Force City-Base, Texas, collects specimens and screens for a variety of viral respiratory pathogens, including influenza A and B, respiratory syncytial virus, adenovirus, and herpes simplex virus (*19,20*). All MTFs are encouraged to submit specimens on a year-round basis, but sentinel sites are specifically directed to submit 6–10 specimens per week during the official influenza season, week 40 in the first year through week 20 in the second year (generally October through early May). The program guidelines state that specimens should only be obtained from patients meeting a clinical case definition of ILI, which at the time of this study was a fever ≥100.5°F (38°C) and either a cough or sore throat (*20*).

We matched individual specimens with outpatient clinic visits that occurred within a 5-day range around the date of specimen collection by using a unique patient code that links the records but does not identify the patient. This analysis included encounters for active duty personnel, dependents, and retirees during the 2-year period of June 2002 to June 2004, but was limited to visits to US Air Force MTFs because we had the ability to link laboratory and outpatient encounter records at these locations. Specimens were first matched to a visit that occurred on the same day that the specimen was collected; those specimens that matched were excluded from subsequent match attempts. Remaining specimens were then sequentially matched to visits 1 day earlier, 1 day later, 2 days earlier, and 2 days later than the date listed as date collected. Upon each iteration of this process, specimens were excluded from the remaining potential match pool if successfully matched to a visit. The purpose of this window approach is to obtain as many matches as possible and allow for some discrepancy between the visit date and the date of collection.

Table 1. Original set of 29 ICD-9 codes included in the influenzalike illness syndrome in ESSENCE*

ICD-9 code	Description	Specificity and severity rank†
079.89	Viral infection NEC	4
079.99	Viral infection NOS	4
460	Nasopharyngitis, acute	4
462	Pharyngitis, acute	4
464.00	Laryngitis, acute, without obstruction	4
464.10	Tracheitis, acute, without obstruction	4
464.20	Laryngotracheitis, acute without obstruction	4
465.0	Laryngopharyngitis, acute	4
465.8	Infectious upper respiratory, multiple sites, acute NEC	4
465.9	Infectious upper respiratory, multiple sites, acute NOS	4
466.0	Bronchitis, acute	3
466.11	Bronchiolitis due to respiratory syncytial virus	3
466.19	Bronchiolitis, acute, due to other infectious organism	3
478.9	Disease, upper respiratory NEC/NOS	4
480.0	Pneumonia due to adenovirus	2
480.1	Pneumonia due to respiratory syncytial virus	2
480.2	Pneumonia due to parainfluenza	2
480.8	Pneumonia due to virus NEC	2
480.9	Viral pneumonia unspecified	2
484.8	Pneumonia in other infectious disease NEC	2
485	Bronchopneumonia, organism NOS	2
486	Pneumonia, organism NOS	2
487.0	Influenza with pneumonia	1
487.1	Influenza with respiratory manifestation NEC	1
487.8	Influenza with manifestation NEC	1
490	Bronchitis NOS	3
780.6	Fever	4
784.1	Pain, throat	4
786.2	Cough	4

*ICD-9, International Classification of Diseases, Ninth Revision; ESSENCE, Electronic Surveillance System for the Early Notification of Community-based Epidemics; NEC, not elsewhere classified; NOS, not otherwise specified.
†Specificity and severity rank: 1, most severe or specific; 4, least severe or specific.

For each encounter linked to a specimen, we selected a single ICD-9 code per individual specimen. Some specimens had more than 1 encounter on the day matched, so we used the following algorithm for selection of the ICD-9 code: if 1 of the ICD-9 codes present was from the ILI syndrome list, it was selected. In cases in which patients had multiple ILI diagnoses, the more specific (for influenza first and other diseases second) or severe code was used, e.g., if both pneumonia and throat pain were included, pneumonia was selected; if pneumonia and influenza with pneumonia were included, influenza with pneumonia was selected (Table 1). If no ILI codes were used for the visit, the code closest to an infectious respiratory diagnosis was used; we gave priority to infectious disease or respiratory codes first, to general symptoms second, to other diagnoses third, and "V codes" (supplementary classification of factors influencing health status and contact with health services) last. We then measured the frequency of positive viral specimens by ICD-9 code.

Trend Analysis of Unmatched Syndromic ICD-9 Codes and DOD Influenza Specimens

A second analysis compared DOD-wide positive specimens from the DOD Global Influenza Surveillance Program to ICD-9 data without matching from October 2000 through December 2004. We extended the date range for this analysis because more data were available for the DOD-wide population. We compared the trend of the DOD-wide specimens to the trends of each individual ICD-9 code in the ILI set, as well as additional codes frequently used in association with the collection of viral specimens in the matched Air Force analysis. We selected individual codes that had trends similar to that of the specimens and evaluated trends for groupings of 3–10 ICD-9 codes. We then measured the association between individual and grouped ILI codes with the positive viral specimens through both standard and lagged correlation analysis. We calculated lagged correlation coefficients by shifting the ICD-9 data by three 1-week increments both forward and backward, while holding the positive specimens constant.

We also performed signal-to-noise analysis of individual codes. First, we defined the influenza season as weeks in which the weekly count of positive specimens was greater than the mean of positive specimens for the study period. We then calculated means and standard deviations of the daily counts for each ICD-9 code. We defined signal as the mean during the influenza season minus the mean during the noninfluenza period and noise as the standard error during the noninfluenza period. The ratio of signal-to-noise evaluated whether individual codes would provide a good signal during the influenza season.

We used 4 separate criteria to select the best performing ICD-9 codes: individual code trend; high correlation coefficient (>0.6 preferable); high signal-to-noise ratio (≥1.5 preferable); and a substantial percentage of positive specimens for either all pathogens (>35%) or influenza virus (>20%). Codes fitting these specifications were retained for further analysis. Because the signal from codes used less often might be lost when combined with more frequently used codes, we created 2 new groupings, 1 with high-volume codes (ILI-large) and 1 with low-volume codes (ILI-small). We defined high-volume codes as being used >50× per day on average or >75,000× during the 4-year study period.

Assessment of Daily Algorithm Performance on ICD-9 Data

We performed another analysis to assess the utility of running daily statistical algorithms on the ESSENCE ILI group, in a way similar to algorithms run on the other 8 syndrome groups. ILI is currently reported as a weekly percentage of visits without statistical alerts. Outbreak detection in ESSENCE is based on a mixed time-series model that combines regression and exponentially weighted moving average (EWMA) algorithms (*10*,*21*,*22*). The number of patient visits is related not only to the previous day's count but also to specific day of the week. The model treats holidays and weekends differently from the days following them. It reduces, or smoothes, artificial peaks in the data, which result not from true epidemics but from surges in patient visits after clinic closures, so that these peaks do not cause frequent false alarms. Likewise, the model accounts for fewer persons seeking care on weekends or during holidays, so these fluctuations do not affect the predictions. For this analysis, we ran the mixed EWMA and regression model on daily counts of the original ESSENCE ILI group, as well as on counts of the new ILI-large and ILI-small groups.

Weekly ILI Trend Comparison between CDC Sentinel Surveillance and DOD ICD-9 Data

From October through May, providers within the US Influenza Sentinel Providers Surveillance Network submit weekly reports to CDC of the total number of patients seen and the number of those patients with ILI (*23*). CDC calculates and reports weekly percentages by region. In this system, ILI is defined as a "fever (temperature of ≥100°F (37.8°C) plus either a cough or a sore throat, in the absence a known cause other than influenza." To confirm the results we found in our comparison of DOD surveillance systems, we analyzed the trends and correlation between weekly DOD-wide ESSENCE ILI groupings and nationwide CDC data during 3 influenza seasons: 2001–02, 2002–03, and 2003–04.

Statistical Analysis

We used Stata version 8.0 (Stata Corporation, College Station, TX, USA) and SAS versions 8.2 and 9 (SAS Institute, Cary, NC, USA) for the direct comparison of specimen data and patient visits and SAS versions 8.2 and 9 for statistical modeling and analysis. The ESSENCE-mixed EWMA and regression models were designed by using SAS macros. This research protocol was approved by the Institutional Review Board at the Walter Reed Army Institute of Research.

Results

During the study period, 7,389 Air Force specimens were taken for the matched analysis. We found an ICD-9–coded visit within the 5-day window surrounding the sample collection date for 6,236 (84.4%), with most of those specimens matching on the exact day (5,267, 84.5%). Of the 6,236 specimens with a match, 339 patients (5.4%) had >1 visit recorded: 321 had 2 visits, 12 had 3 visits, and 1 patient had 4 visits for the same day. Tables 2 and 3 show a breakdown of how the match worked, including multiple visits and multiple ICD-9 codes per visit. We gave preference to the highest order diagnosis for 68 patients who had multiple ILI diagnoses. For the 96 patients who had multiple visits without an ILI code, we selected the closest diagnosis to an infectious disease or one depicting respiratory symptoms.

Table 4 shows the number of specimens associated with each ICD-9 code, as well as the percentage of those specimens that tested positive for any viral respiratory

Table 2. Data showing match of Air Force respiratory virus specimens to ICD-9 coded visits, January 2002–July 2003*

Match day	No. specimens†
Did not match (removed from study)	1,153
Clinic day – 2 = specimen day	47
Clinic day – 1 = specimen day	125
Exact day match	5,267
Clinic day + 1 = specimen day	680
Clinic day + 2 = specimen day	117
Total	7,389

*ICD-9, International Classification of Diseases, Ninth Revision.
†No. specimens obtained by day of matching visit.

Table 3. Data showing match of Air Force respiratory virus specimens to ICD-9 coded visits, January 2002–July 2003*

No. visits/type	No. specimens†
2 visits recorded (n = 321)	
Both non-ILI	90
1 ILI; 1 non-ILI	164
Both ILI	67
3 visits recorded (n = 12)	
3 non-ILI	6
1 ILI; 2 non-ILI	5
2 ILI; 1 non-ILI	1
4 visits recorded (n = 1)	
1 ILI; 3 non-ILI	1

*ICD-9, International Classification of Diseases, Ninth Revision; ILI, influenzalike illness.
†No. specimens obtained by day of matching visit.

pathogen and for influenza virus. We found many of the ILI codes to either be infrequently used with a viral specimen or to have a low percentage of positive specimens. Four codes not in the original ILI group (otitis media, acute suppurative otitis media, acute sinusitis, and acute tonsillitis) were frequently used with the collection of viral specimens.

For the unmatched DOD-wide analysis, we found 15,914 samples taken during the study period, of which 6,340 (39.8%) were positive for any viral respiratory pathogen, and 2,210 (13.9%) were positive for influenza A or B. Temporal analysis showed that as a group, the original ILI syndrome follows the same seasonal pattern as that for positive specimens. Individual ICD-9 code trends for influenza, fever, unspecified viral infection, otitis media, and upper respiratory infection (multiple sites) correlated well with those of the positive specimens (Table 5). Codes that did not correlate with positive specimen trends included acute tonsillitis and throat pain.

Many individual codes that correlated well with the positive specimens also tended to have high signal-to-noise ratios (Table 5). Moreover, the percentage of positive

Table 4. Laboratory specimens matched with outpatient visit ICD-9 data*†

ICD-9 code	Description	No.	% Positive for any viral respiratory pathogen	% Positive for influenza A or B
079.89	Viral infection, NEC*	33	36	33
079.99	Viral infection NOS*	783	51	40
382.00	Otitis media, acute suppurative NOS	30	47	30
382.9	Otitis media NOS	51	31	27
460	Nasopharyngitis, acute	286	36	23
461.8	Other acute sinusitis	0	NA	NA
461.9	Acute sinusitis, unspecified	66	47	28
462	Pharyngitis, acute	637	40	13
463	Acute tonsillitis	57	46	0
464.00	Laryngitis, acute, without obstruction	2	0	0
464.10	Tracheitis, acute, without obstruction	1	0	0
464.20	Laryngotracheitis, acute without obstruction	0	NA	NA
465.0	Laryngopharyngitis, acute	3	67	0
465.8	Infectious upper respiratory, multiple sites, acute NEC	38	68	28
465.9	Infectious upper respiratory, multiple sites, acute NOS	1,251	61	20
466.0	Bronchitis, acute	146	39	15
466.11	Bronchiolitis due to respiratory syncytial virus	33	61	9
466.19	Bronchiolitis, acute, due to other infectious organism	88	30	3
478.9	Disease, upper respiratory NEC/NOS	1	100	0
480.0	Pneumonia due to adenovirus	0	NA	NA
480.1	Pneumonia due to respiratory syncytial virus	2	0	0
480.2	Pneumonia due to parainfluenza	0	NA	NA
480.8	Pneumonia due to virus NEC	6	50	16
480.9	Viral pneumonia unspecified	5	40	40
484.8	Pneumonia in other infectious disease NEC	1	0	0
485	Bronchopneumonia, organism NOS	0	NA	NA
486	Pneumonia, organism NOS	238	40	12
487.0	Influenza with pneumonia	4	100	75
487.1	Influenza with respiratory manifestation NEC*	372	54	49
487.8	Influenza with manifestation NEC	46	46	43
490	Bronchitis NOS	26	39	26
780.6	Fever	611	74	13
784.1	Pain, throat	14	14	0
786.2	Cough	52	37	23

*International Classification of Diseases, Ninth Revision (ICD-9); NOS, not otherwise specified; NEC, not elsewhere classified (as listed in the ICD-9); NA, not assessed.
†Laboratory specimen data matched with outpatient visit data in Air Force data analysis, June 2001–June 2003.

Table 5. Unmatched outpatient visit data and final influenzalike illness (ILI) syndromic groupings

	Unmatched data†					ILI group	
ICD-9 code*	Total volume of code use (2001–04)	Average daily count	Correlation with positive specimens	p value	Signal-to-noise ratio	Original	New (final)
079.89	17,729	11	0.3355	<0.0001	1.05	Yes	-
079.99	1,115,143	718	0.7746	<0.0001	2.84	Yes	Large
382.00	277,270	179	0.4868	<0.0001	1.58	No	-
382.9	1,185,809	764	0.6286	<0.0001	1.76	No	Large
460	361,139	233	0.5552	<0.0001	1.55	Yes	Large
461.8	123,913	80	0.3083	<0.0001	1.14	No	-
461.9	741,085	477	0.6017	<0.0001	1.81	No	Large
462	1,436,325	925	0.5468	<0.0001	1.61	Yes	-
463	168,499	108	0.3176	<0.0001	0.08	No	-
464.00	22,470	14	0.1133	0.1085	0.14	Yes	-
464.10	1,736	1	0.2560	0.0002	0.56	Yes	-
464.20	3,539	2	0.3852	<0.0001	0.88	Yes	-
465.0	33,760	22	0.4804	<0.0001	1.11	Yes	-
465.8	72,042	46	0.6384	<0.0001	1.86	Yes	Small
465.9	3,989,688	2,569	0.6758	<0.0001	1.91	Yes	Large
466.0	632,256	407	0.6693	<0.0001	2.17	Yes	Large
466.11	18,377	12	0.4800	<0.0001	1.83	Yes	-
466.19	68,127	44	0.5257	<0.0001	1.65	Yes	-
478.9	7,434	5	0.4296	<0.0001	1.23	Yes	-
480.0	287	0	0.0889	0.2082	0.24	Yes	-
480.1	1,790	1	0.4083	<0.0001	1.58	Yes	-
480.2	451	0	0.3316	<0.0001	1.64	Yes	-
480.8	11,708	8	0.3501	<0.0001	1.07	Yes	-
480.9	10,852	7	0.4562	<0.0001	1.44	Yes	-
484.8	4,312	3	0.3202	<0.0001	1.11	Yes	-
485	7,954	5	0.4180	<0.0001	0.99	Yes	-
486	322,397	208	0.7180	<0.0001	2.09	Yes	Large
487.0	5,093	3	0.6205	<0.0001	3.11	Yes	Small
487.1	62,340	40	0.8696	<0.0001	5.59	Yes	Small
487.8	8,973	6	0.7926	<0.0001	4.74	Yes	Small
490	297,918	192	0.6337	<0.0001	1.50	Yes	Large
780.6	470,770	303	0.7545	<0.0001	2.55	Yes	Large
784.1	59,516	38	0.2994	<0.0001	0.59	Yes	-
786.2	545,510	351	0.5573	<0.0001	1.24	Yes	Large

*See Table 4 for description of International Classification of Diseases, Ninth Revision (ICD-9) code.
†Department of Defense–wide outpatient visit data, October 2000–December 2004.

specimens associated with many of these codes also tended to be high (Table 4). Based on the results of these 3 tests and their individual trends, we selected 14 ICD-9 codes for ILI surveillance. We used the frequency of individual code use during the 4-year analysis period to group 10 of the 14 codes into the ILI-large group and the other 4 into the ILI-small group, as indicated in Table 5.

Lagged correlation analysis found that the codes of both subsets tend to peak at the same time as the number of positive specimens (Figure 1). However, the ILI-Small group codes, while still peaking centrally, tended to have curves slightly skewed to the right in the lagged correlation plot, indicating that they may be more likely to follow, rather than predict, the increases in ILI visits.

After establishing the new small and large ILI groups, we found that the weekly temporal trends closely follow those of positive respiratory specimens (Figure 2). Correlation coefficients of the weekly data were 0.72 ($p<0.0001$), 0.71 ($p<0.0001$), and 0.86 ($p<0.0001$) for the original, ILI-large, and ILI-small groups, respectively.

We ran the EWMA/regressive model on 4 years of daily DOD outpatient data in each of the 3 comparison groups (Figure 3). Multiple seasonal outbreaks of respiratory illness were identified with alerts for all groupings. The daily algorithm triggered alerts much more frequently on the ILI-small group than on the large group; the algorithm for the small grouping tended to be more responsive to smaller fluctuations in the data.

Direct comparison of the nationwide US Influenza Sentinel Providers Surveillance Network with the ESSENCE ILI groupings showed very similar trends during each of the previous 3 seasons (Figure 4). Further analysis showed that CDC data were very strongly correlated with data from the ILI-small group; with correlation

coefficients 0.97 ($p<0.0001$), 0.87 ($p<0.0001$), and 0.99 ($p<0.0001$) for the 2001–02, 2002–03, and 2003–04 seasons, respectively. Correlation coefficients for the ILI-large group were also very strong, although not quite as high: 0.88 ($p<0.0001$), 0.77 ($p<0.0001$), and 0.93 ($p<0.0001$), respectively.

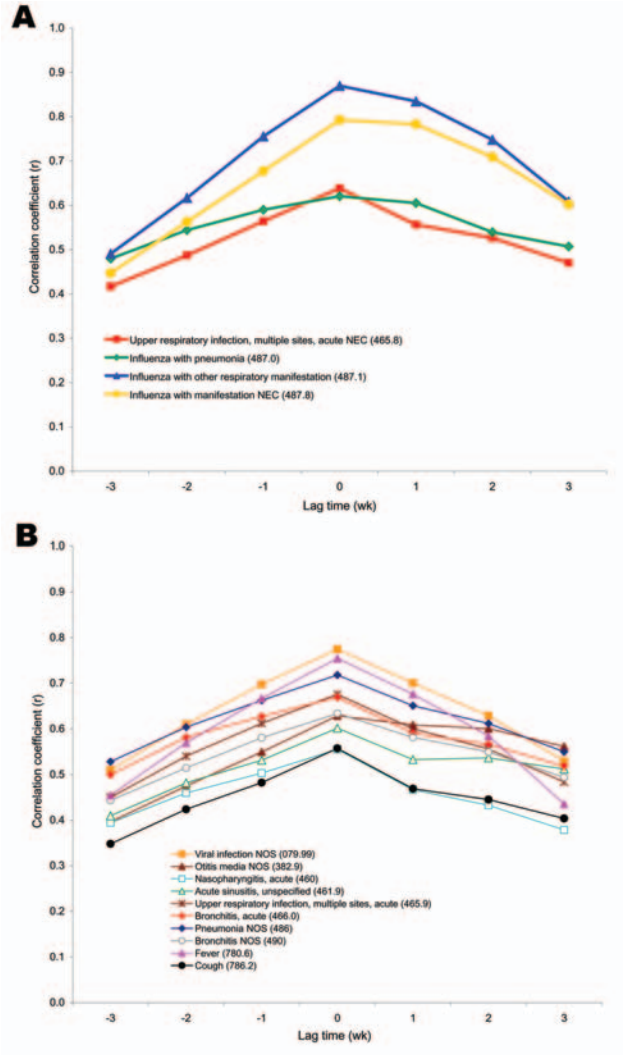

Figure 1. Lagged correlation analysis between individual codes from the International Classification of Diseases, Ninth Revision (ICD-9), and unmatched positive respiratory specimens from October 2000 to December 2004. Each of the individual ICD-9 codes that had high signal-to-noise ratio and high correlation when compared with positive influenza laboratory specimens taken during the same time frame (Table 5) were compiled into new large and small influenzalike illness (ILI) groups (large codes were used >50×/day on average) and compared again to the positive specimens through lagged analysis. The ICD-9 data were shifted by three 1-week increments both forward and backward, while holding the positive specimens constant. A) Lag time correlation coefficients for ICD-9 codes in the new large count ILI group. B) Lag time correlation coefficients for ICD-9 codes in the new small count ILI group. NEC, not elsewhere classified; NOS, not otherwise specified.

Discussion

In our experience with ESSENCE, the ILI surveillance report has been one of the most useful components. Military public health officials, and now some civilian health departments, use ESSENCE to monitor the ILI grouping for early signs of the influenza season and other common febrile respiratory outbreaks. In a similar manner, CDC now monitors ILI by using the same DOD data within the BioSense system. This study shows that the DOD outpatient ICD-9 data are indeed useful and accurate for routine influenza surveillance.

Critical analysis of the ICD-9 codes within the ESSENCE ILI group showed that approximately half of the codes were associated with specimens positive for respiratory pathogens, including influenza. Temporal trends confirmed that most codes followed the same trends over time as positive specimens. Codes with low correlation to

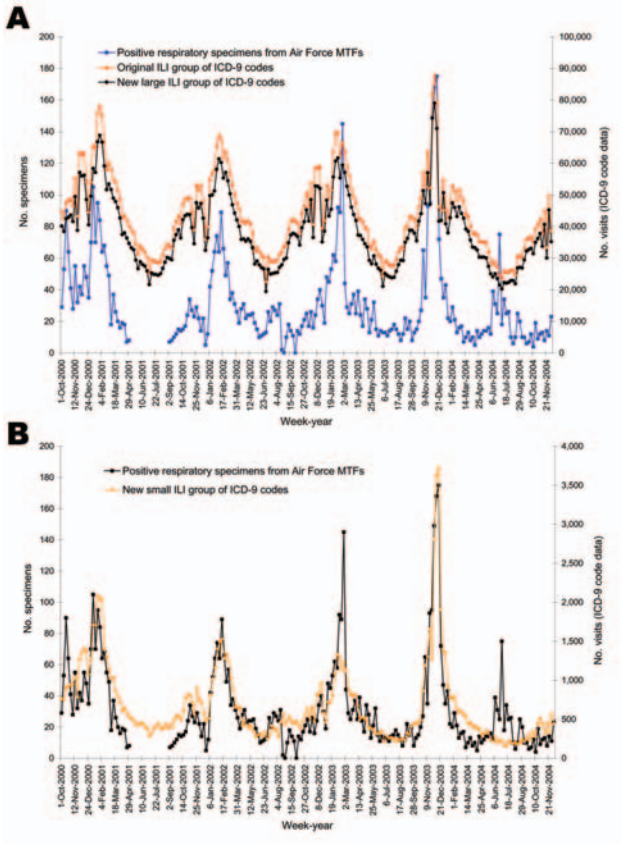

Figure 2. Weekly trends among unmatched visits coded by International Classification of Diseases, Ninth Revision (ICD-9), and specimens positive for any viral respiratory pathogen from October 2000 to December 2004. Based on correlation to positive specimens and signal-to-noise ratios, new large and small influenzalike illness (ILI) categories were created. The number of positive specimens is depicted on the left y-axis and compared to the number of visits for the original, new large and new small ILI ICD-9 categories, as shown in the right y-axis. A) Original ILI and new ILI-large groups with positive specimens. B) New ILI-small group with positive specimens. MTFs, military treatment facilities.

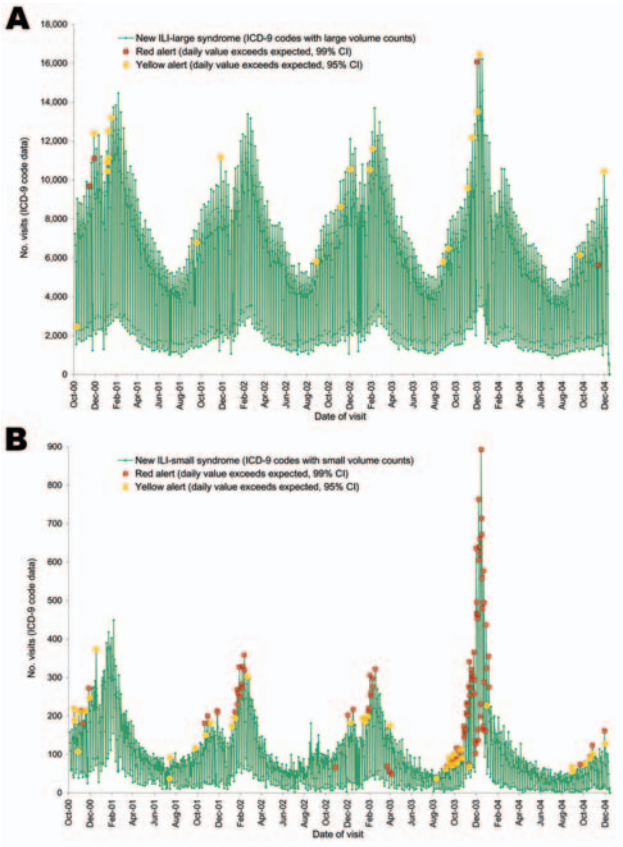

Figure 3. Newly created groups of International Classification of Diseases, Ninth Revision (ICD-9), codes for influenzalike illness (ILI) based on correlation to positive specimens and signal-to-noise ratios were run with anomaly detection algorithms. Two groups, large and small, were created with ICD-9 codes that had an average use of >50× per day in the large group with the remainder in the small group. Daily counts of the codes in the large and small syndromic groups were plotted from October 2000 to December 2004. An algorithm based on a mixed time series model that combines regression and exponentially weighted moving average (EWMA) is used to detect potential outbreaks and takes into account weekends and holidays. Yellow alerts occur when the daily value exceeds that expected with a 95% confidence interval, and red alerts occur when the amount exceeds the expected with a 99% confidence interval. A) Large syndrome group. B) Small syndrome group. CI, confidence interval.

positive specimens and different temporal trends have been removed from the group to produce more parsimonious groups. The less-specific ILI-large group may be more useful for the initial detection of influenza season and for detecting other respiratory illnesses that initially cause similar symptoms, whereas the ILI-small group is more specific but also more likely to signal slightly later than the large group because providers may use these codes cautiously until influenza cases have been confirmed. However, both groupings have been shown to be useful indictors of an impending influenza season.

ESSENCE should produce reports of ILI activity faster than both the laboratory-based DOD Global Influenza Surveillance Program and the CDC sentinel ILI system because it is able to collect and analyze data more rapidly than specimens and provider reports can be processed. The weekly data are reported in ESSENCE immediately on completion of a full week, whereas the DOD laboratory data have an inherent lag time because of the time required for specimen shipping, laboratory testing, analysis, and reporting. The CDC sentinel reporting system similarly lags behind because of the passive nature of data collection and additional time required to compile and post results. The automated data collection also allows for the potential to analyze data more frequently than the current weekly standard. Our analysis successfully identified seasonal outbreaks by using a combination algorithm on daily data, based on aggregated data for a given day. The algorithm runs every 8 hours (more or less frequently depending on administrator settings) and recalculates on the basis of newly received data. Daily detection algorithms can be instituted on the large and small groups simultaneously to best detect ILI outbreaks.

The results of this study support previous findings on the ability of automated systems to capture the same trends as traditional surveillance. The Minnesota Department of Health found that an ILI grouping of ICD-9 data from a health maintenance organization in the Minneapolis–St. Paul area correlated with reported deaths from pneumonia and influenza (*24*). Ambulatory ICD-9 codes were also successfully used for surveillance of respiratory illnesses in Massachusetts and were highly correlated with hospital admissions that had a lower proportion of discharged

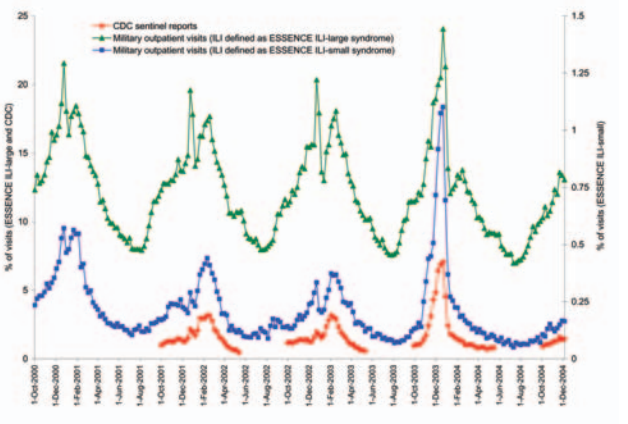

Figure 4. Percentage of visits for influenzalike illness (ILI) using both the large and small syndrome groups among military outpatient visits nationwide compared with Centers for Disease Control and Prevention (CDC) sentinel clinician reports from October 2000 through December 2004. Data are grouped weekly from Sunday through Saturday. CDC data are only obtained during the influenza season. ESSENCE, Electronic Surveillance System for the Early Notification of Community-based Epidemics.

patients with a diagnosis of respiratory illness (*25*). Our study also supports evidence that using nontraditional electronic data for syndromic surveillance may enable health providers to recognize and detect the influenza season faster than with traditional means. In a similar study of nontraditional data, the New York City Department of Health and Mental Hygiene reported that their syndromic system, based on chief complaints at emergency departments, detected the first citywide signs of influenza activity sooner than laboratory- and sentinel-based surveillance (*26*).

We have established that ICD-9–based surveillance that uses the ILI-large and ILI-small groups is an effective tool for influenza surveillance. We suggest that health agencies use these syndrome groups as a model for developing similar systems. However, we strongly emphasize that developers perform critical analysis of the individual codes collected in their data and carefully consider not only the clinical basis for code inclusion but also which diagnoses are more likely to cause background "noise" rather than contribute to the signal. Our own evaluation illustrates the importance of such critical review, as we found that both throat pain and acute tonsillitis had more noise than signal. Asthma and chest pain are included in other syndromic systems (*24*); however, in the DOD data, these tend to occur year-round with fairly high volume and contribute more noise than signal in the DOD ambulatory data. Studies of systems that use such broad categories for ILI surveillance have yielded lower correlation of ICD-9 data with mortality and laboratory-based data (*24*). Data sources differ dramatically in population coverage, quality and accuracy, and most important, in their ability to reflect true disease patterns. Our method for defining and assessing syndrome groupings for ICD-9–based surveillance should assist developers in parsing, analyzing, and interpreting their own data.

Ms Marsden-Haug is currently an epidemiologist for the Tacoma-Pierce County Health Department (TPCHD). She evaluates syndromic surveillance systems used by TPCHD and the Washington State Department of Health, and assists with other surveillance projects for the TCPHC Communicable Disease Control unit.

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Address for correspondence: Julie A. Pavlin, Department of Microbiology and Immunology, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD 20814, USA; email julie.pavlin@us.army.mil

Imported Infectious Disease and Purpose of Travel, Switzerland

Lukas Fenner,*[1] Rainer Weber,* Robert Steffen,* and Patricia Schlagenhauf*

We evaluated the epidemiologic factors of patients seeking treatment for travel-associated illness from January 2004 through May 2005 at the University Hospital of Zurich. When comparing persons whose purpose of travel was visiting friends and relatives (VFR travelers; n = 121) with tourists and other travelers (n = 217), VFR travelers showed a distinct infectious disease and risk spectrum. VFR travelers were more likely to receive a diagnosis of malaria (adjusted odds ratio [OR] = 2.9, 95% confidence interval [CI] 1.2–7.3) or viral hepatitis (OR = 3.1, 95% CI 1.1–9) compared with other travelers but were less likely to seek pretravel advice (20% vs. 67%, p = 0.0001). However, proportionate rates of acute diarrhea were lower in VFR (173 vs. 364 per 1,000 ill returnees). Travel to sub-Saharan Africa contributed most to malaria in VFR travelers. In countries with large migrant populations, improved public health strategies are needed to reach VFR travelers.

More than 800 million tourist arrivals were registered worldwide in 2005, and an estimated 2% of the world's population lives outside the country of birth (*1*). Importation of infectious diseases to new countries is likely to increase among both travelers and immigrants. Approximately 80 million people from resource-rich areas worldwide travel to resource-poor countries every year (*2*) and are exposed to many infections that are no longer prevalent in the countries where they live. Travelers visiting friends and relatives (VFR travelers)—predominantly immigrants and their children returning to their home countries for vacations, to maintain family ties, or to visit sick relatives—are at particularly high risk for preventable infectious diseases, such as malaria, typhoid fever, hepatitis A, hepatitis B, and tuberculosis (*3–5*).

A recent review of a global surveillance network's data set showed different demographic characteristics and different types of travel-related illnesses among immigrant-VFR, traveler-VFR, and tourist travelers (*5*). The population of western Europe includes ≈20 million persons living in nonnative countries; most are settled immigrants. One third were born in a country outside of Europe (*6*). In Switzerland, ≈21% (1.6 million) residents are foreign born (*7*). Compared with the health of the native population of Switzerland, the health status of the immigrant population is poor (*8*) because of the high prevalence of infectious diseases in the home countries (*9*), a difficult psychosocial environment in the new country, inappropriate risk-taking behavior (*10*), and social inequalities (*11*).

The University Hospital of Zürich serves a large proportion of the city's population, which includes a multiethnic range of patients and immigrants. The outpatient departments treat ≈120,000 patients each year, and the inpatient departments treat >35,000. We evaluated the epidemiology of imported infectious disease of patients seeking treatment for travel-associated illness at the University Hospital of Zürich from January 2004 through May 2005.

Patients and Methods

The University Hospital of Zürich, as part of the global GeoSentinel surveillance network, contributed clinician-based surveillance data during a 17-month period, January 2004–June 2005, according to demographic characteristics, risk for infectious disease while traveling, and frequency of pretravel advice. GeoSentinel is a global sentinel surveillance network that was established in 1995 through the International Society for Travel Medicine and the US Centers for Disease Control and Prevention. The network consists of 33 globally distributed member travel/tropical medicine clinics (*12*) and has been widely used to document travel-related illnesses (*5*,*13–15*).

*University of Zürich, Zürich, Switzerland

[1]Current affiliation: University Hospital Basel, Basel, Switzerland

Inclusion Criteria

To be eligible, patients must have crossed an international border ≤10 years before seeking treatment and must have sought medical advice for a presumed travel-related illness. Relevant travel details focused only on data from the 6 months before the onset of illness. Only final diagnoses were considered, and >1 diagnosis per patient was possible. Data were collected according to a standardized, anonymous questionnaire. The questionnaire asked for demographic data (age, sex, country of birth, country of residence, current citizenship), travel history during the previous 5 years, inpatient or outpatient status, major clinical symptoms (>1 per patient possible), pretravel visit information, reason for most recent travel, and patient classification. Reasons for most recent travel were immigration, tourism, business, research/education, missionary/volunteer work, visit to friends or relatives, and expatriation. Patients were classified as immigrants/refugees, foreign visitors, urban expatriates, nonurban expatriates, students, military personnel, or travelers. Working and final diagnoses were assigned by a physician.

Definitions

An immigrant/refugee was defined as a foreign-born person who had obtained permanent resident status or immigrant/refugee status in Switzerland. Traveler (or traditional traveler) was defined as a resident of Switzerland who crossed an international border and did not previously immigrate to Switzerland. When the purpose of recent travel was visiting friends and relatives, a traveler was termed VFR. Different patient classifications were possible (i.e., immigrant-VFR, traveler-VFR). The rate of illness was calculated as the number of patients with a specific or a summary diagnosis as a proportion of all VFR or traditional travelers, respectively, expressed as number per 1,000 patients. The percentage of "chief complaints" was expressed as the number of primary symptoms that led to a clinic visit per total patients in each group. More than 1 chief complaint per patient was possible.

Countries were assigned to 1 of 15 regional classifications (*13*). Because of small case numbers, a more simplified regional classification was sometimes used: sub-Saharan Africa, south-central America (South and Central America), Asia (south-central, southeast, east, and north Asia), and eastern Europe. "All other regions" include those with no assigned travel destination. For travelers or VFR who entered >1 region, the most likely place of exposure during travel was determined to be the single region visited.

Summary diagnosis were defined as follows: "respiratory tract infection" included upper and lower respiratory infections; "malaria" infections included all malaria-causing species; "diarrhea" included acute diarrhea of parasitic, viral, bacterial or unknown origin; "hepatitis" included chronic or acute viral hepatitis; "viral syndrome" included any nonspecific viral symptoms; and "AIDS/HIV/STI" included asymptomatic HIV, acute HIV, AIDS, gonorrhea, syphilis, and other sexually transmitted infections (STIs). Syndrome groups such as "dermatologic disorder" were defined as previously described (*15*).

Statistics

Stata software (version 9.1, Stata Corporation, College Station, TX, USA) was used for statistical analysis. Odds ratios (OR) of binary, categorical, or continuous variables were determined by logistic regression (multivariate or univariate) and adjusted to age and sex if indicated. Statistical significance of dichotomous variables was achieved by using χ^2 or nonparametric tests.

Results

General Description and Demographic Data

We analyzed 451 patients included in the database: 181 immigrants, 227 travelers, 25 foreign visitors, and 18 others (expatriates, students, military personnel). Age range was 16–87 years (median 33, interquartile range 27–43); 48% were female, and 20% were inpatients. The median duration of travel was 17.5 days (interquartile range 13–29 days). For these patients, 671 diagnoses were counted. Leading complaints were "fever" (43.0%), "gastrointestinal" (42.7%), "head-ear-nose" (25.2%), "respiratory" (24.3%), "musculoskeletal" (12.8%), and "skin" (11.9%, data not shown). The visits were evenly distributed during the calendar year, with no seasonal abnormities or significant associations.

Comparison of VFR and Traditional Travelers

Our analysis included 217 traditional travelers and 121 VFR travelers. For traditional travelers, the reason for most recent travel was tourism or business. Most VFR travelers (86%) were in the category "immigrants." Birth country regions of VFR travelers were Asia (30%), sub-Saharan Africa (24%), Eastern Europe (17%), and Central or South America (11%). The basic demographic pattern was comparable (Table 1). VFR travelers traveled on average for a longer period than traditional travelers, were slightly older, were more likely to have inpatient status, and were less likely to seek pretravel advice. Traveled regions were also comparable (Table 1). Fever and gastrointestinal disorders were the most frequent reasons for seeking treatment (Table 2). Traditional travelers had more gastrointestinal symptoms (53.91% vs. 39.66%, $p = 0.03$). When the disease spectrums were compared, acute diarrhea was more often diagnosed in traditional travelers (26%) than in VFR travelers (11%). The summary diagno-

Table 1. Demographic data on persons included in the study whose purpose of travel was visiting friends and relatives (VFR) versus traditional travelers (travelers), Switzerland

	Travelers, no. (%), n = 217	VFR, no. (%), n = 121	p value
Sex			
Male	119 (54.8)	61 (50.4)	0.43
Female	98 (45.2)	60 (49.6)	
Age (y)			
Median	32	39	0.008
Interquartile range	32–46	26–45	
Patient type			
Outpatient	185 (84.5)	84 (70.6)	0.002
Inpatient	34 (15.5)	35 (29.4)	
Travel duration (d)			
Median	15	21	0.006
Interquartile range	11–24	14–31	
Sought pretravel advice?			
Yes	65 (67)	18 (20)	0.0001
No	32 (33)	70 (80)	
Traveled region			
Sub-Saharan Africa	43 (19.81)	27 (22.31)	
Asia	61 (28.11)	21 (17.35)	
Eastern Europe	6 (2.76)	21 (17.35)	
Central/South America	22 (10.13)	9 (7.43)	
All other regions	85 (39.17)	43 (35.53)	

sis HIV/AIDS/STI was more commonly established in VFR travelers (9.9% vs. 4.3%); the same was true for malaria (7.7% vs. 2.7%). The proportionate illness patterns are shown graphically in the online Appendix Figure (available from www.cdc.gov/EID/content/13/2/217-appG.htm).

When comparing VFR with traditional travelers, VFR travelers were more likely to receive a diagnosis of malaria, acute or chronic viral hepatitis, and HIV/AIDS/STI (Table 3) but less likely to receive a diagnosis of acute diarrhea. In contrast, traditional travelers were more likely to receive a diagnosis of diarrhea (OR 2.1, 95% confidence interval [CI] 1.2–3.6, p = 0.007; data not shown). Respiratory diseases and viral syndromes were significantly associated with VFR travelers only in the univariate analysis (Table 3). Traditional travelers were significantly more likely to seek pretravel advice compared with VFR travelers (Table 1).

A different infectious disease spectrum and a trend toward a distinct pattern in both VFR and traditional travelers were also found when selecting different travel regions (Figure). Malaria cases were almost exclusively imported from the sub-Saharan Africa region; 33.3% of diagnoses after travel to this region were attributed to malaria in VFR travelers, compared with 12.3% in traditional travelers. In total, 27 malaria cases were recorded in the GeoSentinel database during the 17-month period: 14 in VFR travelers, 8 in tourist travelers, 4 in recent immigrants, and 1 in an immigrant/refugee. Of these, 22 cases were imported from sub-Saharan Africa and 1 from Turkey; for 4 case-patients, no specified travel region or no information on place of exposure was available. When data were stratified by VFR versus traditional traveler, the risk for malaria in sub-Saharan Africa was twice as high in the VFR traveler group than in the traditional traveler group (data not shown).

Discussion

The GeoSentinel site based at the University Hospital of Zürich represents a large population in Switzerland. However, GeoSentinel is a health facility–based surveillance system and does not actively screen for certain diseases. Patients included in the database do not necessarily represent the whole population or the epidemiology or frequency of the disease. Besides the unknown number of ill returned travelers going to general practitioners or nonspecialized clinics, the number of travelers returning in good health is also unknown. Incidence rates or relative risks therefore cannot be estimated. Similarly, patients with mild or self-limiting disease are likely to see a general practitioner rather than to go to a specialized center, although many VFR travelers do not have a regular general practitioner. On the other hand, Zürich is a large city with a socioculturally mixed population that offers an opportunity to study immigrant-VFR travelers, and many of these patients may prefer to go to a more anonymous university hospital than to a general practitioner. A limitation of the study is the relatively small number of patients included in the database during the 17-month period, which made it necessary to form summary diagnoses and regions.

In our analysis, VFR travelers showed a different infectious disease and risk spectrum than did traditional

Table 2. Primary symptoms of persons seeking treatment at a clinic, frequent summary diagnosis, and syndrome groups in persons whose purpose of travel was visiting friends and relatives (VFR) versus traditional travelers (travelers), Switzerland

	Travelers, no. (%)*	VFR, no. (%)*
Primary symptom		
Fever	108 (49.76)	57 (47.10)
Gastrointestinal	117 (53.91)	48 (39.66)
Head-ear-nose	54 (24.88)	38 (31.40)
Respiratory	52 (23.96)	34 (28.09)
Musculoskeletal	25 (11.52)	22 (18.18)
Skin	30 (13.82)	14 (11.57)
Fatigue	24 (11.05)	13 (10.74)
Other	18 (8.29)	16 (13.22)
Total	428	242
Summary diagnosis and syndrome groups		
Diarrhea, acute	79 (26.33)	21 (11.53)
Respiratory infection	40 (13.33)	22 (12.09)
HIV/AIDS	12 (4)	15 (8.24)
Malaria, all species	8 (2.67)	14 (7.69)
Viral syndrome	23 (7.67)	10 (5.49)
Viral hepatitis, acute/chronic	6 (2)	10 (5.49)
Urinary tract infection	3 (1)	3 (1.65)
Febrile illness, unspecified	10 (3.33)	1 (0.55)
Dengue fever (uncomplicated)	4 (1.33)	1 (0.55)
Sexually transmitted infection	1 (0.33)	3 (1.65)
Loa loa	–	2 (1.1)
Cutaneous leishmaniasis	1 (0.33)	–
Typhoid/paratyphoid fever	1 (0.33)	1 (0.55)
Brucellosis	–	1 (0.55)
Extraintestinal amebiasis	1 (0.33)	–-
Dermatologic disorder	22 (7.33)	9 (4.95)
Chronic diarrhea	7 (2.33)	5 (2.75)
Healthy	4 (1.33)	2 (1.1)
Adverse drug or vaccine reaction	3 (1)	1 (0.55)
Cardiovascular disorder	2 (0.67)	3 (1.65)
Neurologic disorder	2 (0.67)	2 (1.1)
Lost to follow-up	2 (0.67)	–
Pulmonary embolism	1 (0.33)	2 (1.1)
Psychological disorder	1 (0.33)	2 (1.1)
Death	1 (0.33)	1 (0.55)
Other diagnosis	66 (22)	51 (28.02)
Total	300	182

*Percentage expressed as number of primary symptoms that led to a clinic visit per total patients in each group.

travelers; were more likely to receive a diagnosis of malaria, viral hepatitis, or HIV/AIDS/STI; and were less likely to seek pretravel advice. Traditional travelers (mainly tourists) were significantly more likely to seek advice before traveling and to have a posttravel diagnosis of acute diarrhea. This is consistent with previous studies from European migrants returning to their home countries (*16*), as well as a recent review of the global GeoSentinel database (*5*). Malaria is most likely to be acquired in the sub-Saharan Africa region, according to our data and those of others (*13*,*15*).

By contrast, acute diarrhea was the greatest problem in traditional travelers, with an illness rate of 364 per 1,000 ill returned travelers compared with 173/1,000 in VFR travelers. Acute diarrhea, or traveler's diarrhea, is known to affect >50% of travelers, depending on the destination (*17*). The protective effect in VFR travelers could reflect immunity due to recent exposure or exposure in childhood.

Acute or chronic viral hepatitis was also significantly associated with VFR travel, which correlates with a recent study of hepatitis A virus infections in Swiss travelers during a period of 12 years that identified VFR travelers as a high-risk group, especially children of immigrants (*18*). Other significant associations of disease between VFR and traditional travelers were not found; however, this does not necessarily mean that no such relationship exists.

Systemic febrile illnesses, including malaria and typhoid fever, tuberculosis, and respiratory syndromes, are more frequently diagnosed among VFR travelers (*5*). In our study, respiratory diseases contributed to the relatively

Table 3. Association of infectious disease in persons returning to Switzerland whose purpose of travel was visiting friends and relatives versus traditional travelers*

	Odds ratio	p value	95% CI
Univariate analysis			
HIV/AIDS/STI	2.42	0.019	1.15–5.07
Malaria	3.04	0.014	1.25–7.40
Diarrhea, acute	0.36	0.0001	0.21–0.61
Viral hepatitis	2.84	0.046	1.01–7.97
Respiratory infection	0.89	0.692	0.51–1.55
Viral syndrome	0.7	0.362	0.32–1.50
Multivariate analysis (adjusted to age and sex)			
HIV/AIDS/STI	2.63	0.014	1.21–5.69
Malaria	2.93	0.021	1.17–7.32
Diarrhea, acute	0.47	0.007	0.27–0.81
Viral hepatitis	3.15	0.032	1.10–9.02

*CI, confidence interval; STI, sexually transmitted infection.

high rate of illness in both VFR and traditional travelers (181 vs. 184 per 1,000 ill returnees). No significant association could be established between influenza, long trip duration, and travel involving visiting friends and relatives as described before (*14*), probably because of small numbers and very few cases of influenza. Viral syndrome, a rather loosely defined summary diagnosis with unspecific viral symptoms, was also frequently diagnosed and can be interpreted as a flulike syndrome. Other typical tropical infectious diseases, such as typhoid fever, leishmaniasis, dengue fever, or brucellosis, were rarely diagnosed.

This study shows that VFR travelers are at greater risk for certain infectious diseases and have a disease spectrum distinct from that of traditional travelers. Malaria is the most important, life-threatening imported disease for both nonimmune and VFR travelers, and malaria acquisition is even more likely in VFR travelers. For other infectious diseases, HIV and STIs must also be included in the differential diagnosis, particularly for VFR travelers. VFR travelers are vulnerable because they may visit more rural destinations, live under poor sanitary conditions, and stay away for longer periods (*3*,*4*). Moreover, the health condition of the immigrant population in Switzerland is poor compared with that of the native population (*8*). Prevalence gaps in disease and disparities in access to care exist not only between countries but also between population groups within countries.

In addition, VFR travelers often did not seek pretravel advice. Thus, culturally sensitive strategies for pretravel contact with VFR travelers are greatly needed. Further surveillance of traveler groups with denominator data is needed, and prospective studies focusing on behavioral aspects of disease prevention would allow for evidence-based interventions as part of a public health strategy.

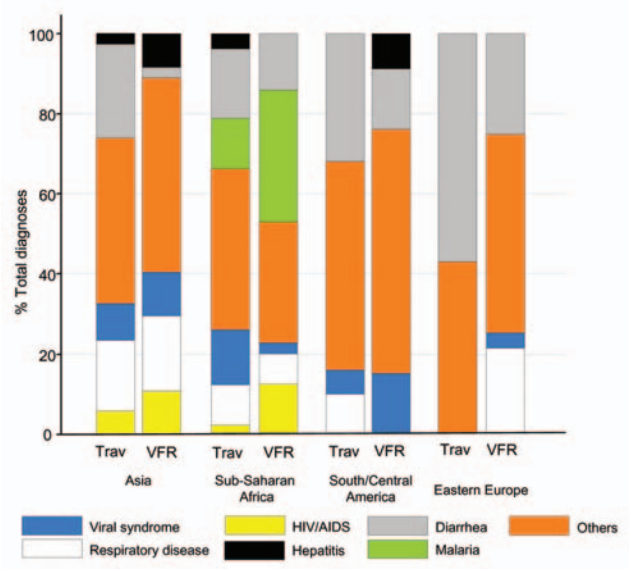

Figure. Percentage of disease diagnoses in travelers visiting friends and relatives (VFR) and traditional travelers (trav) who reported illnesses after returning to Switzerland, classified by geographic region visited.

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Dr Fenner obtained his medical degree from the Medical Faculty of Basel, Switzerland, and is resident microbiologist at the University Hospital, Basel. His research interests include international health and infectious diseases epidemiology.

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Address for correspondence: Patricia Schlagenhauf, University of Zürich Centre for Travel Medicine, World Health Organization Collaborating Centre for Travellers' Health, University of Zürich, Hirschengraben 84, CH-8001 Zürich, Switzerland; email: pat@ifspm.unizh.ch

Invasive Group B Streptococcal Infection in Infants, Malawi

Katherine J. Gray,* Sally L. Bennett,† Neil French,* Amos J. Phiri,* and Stephen M. Graham*†

Group B streptococci (GBS) are a recently identified cause of neonatal sepsis in Malawi. In Queen Elizabeth Central Hospital, Blantyre, Malawi, during May 2004–June 2005, GBS were isolated from routine blood and cerebrospinal fluid cultures from 57 infants. The incidence of early (EOD) and late onset (LOD) invasive GBS disease was 0.92 and 0.89 cases per 1,000 live births, respectively. Sepsis (52%) was the most common manifestation of EOD; meningitis (43%) and sepsis (36%) were the principal manifestations of LOD. The case-fatality rate was 33% overall (38% EOD, 29% LOD). Serotypes Ia and III were responsible for 77% of disease. All isolates were susceptible to penicillin, but 21% were resistant to erythromycin. The rate and manifestations of neonatal GBS disease in Malawi are similar to those in industrialized countries, but the case-fatality rate is higher than in industrialized countries. Effective locally relevant prevention strategies are needed.

Group B streptococcus (GBS) has been a leading cause of neonatal illness and death in many parts of the world, especially industrialized countries, for several decades (*1–5*). In contrast, until recently GBS was infrequently reported in the developing world. A World Health Organization multicenter study of the bacterial etiology of serious infections in young infants of <3 months of age reported in 1999 that the "virtual absence of GBS was striking" (*6*). Yet the prevalence of maternal carriage of GBS in developing countries, including populations in tropical Africa, is similar to that identified in populations in the United States (*7–9*). Recent studies from Kenya (*10–12*), South Africa (*13,14*), Zimbabwe (*15*), and Malawi (*16*) suggest that GBS is emerging as an important cause of neonatal sepsis in Africa. The largest of these studies reported that 136 of 801 bacterial isolates from 784 Malawian neonates were GBS, which makes it the most common cause of sepsis among neonates admitted to Queen Elizabeth Central Hospital (QECH) in Blantyre (*16*).

Prevention strategies such as chemoprophylaxis are available for neonatal GBS but are difficult to apply in a resource-limited setting (*4,5*). Vaccination is an attractive option in this setting, and vaccines consisting of GBS capsular polysaccharide conjugated to a tetanus toxoid carrier protein have been under development (*17–20*). The vaccines are immunogenic in women but of unproven clinical benefit. Important information to support future preventive strategies includes estimate of rates of disease, timing of disease initial manifestations; and for vaccine development, description of serotype distribution in different populations (*5*). Therefore, we set out to further characterize GBS disease in Blantyre District in Malawi.

Methods

Study Setting

The study was conducted during 14 months from May 1, 2004, to June 30, 2005, at QECH in Blantyre District. This district has the largest urban population in Malawi, and much of the population lives in impoverished townships. The predicted midyear population in 2005 was 1,070,173 (www.nso.malawi.net). This estimate is based on projections from the 1998 national census. QECH is an urban district hospital, which takes direct admissions and referrals from surrounding district health centers. It is the only major hospital providing free care in Blantyre. Birth and death statistics for Blantyre for the study period were obtained directly from QECH and the Blantyre District Health Office.

*Malawi-Liverpool–Wellcome Trust Programme of Clinical Tropical Research, Blantyre, Malawi; and †College of Medicine, Blantyre, Malawi

Study Population

Neonates (birth to 6 days of age) are normally admitted directly to the neonatal nursery from the labor ward or postnatal wards. Neonates may also be referred from surrounding health centers in Blantyre District if problems occur immediately after delivery. Young infants from birth to 6 months of age (including those from birth to 90 days of age) who were discharged well after delivery at QECH or in peripheral health centers but in whom symptoms suggestive of sepsis subsequently developed are normally admitted to the pediatric ward. What proportion of infants with sepsis in Blantyre is seen at healthcare facilities is not known.

Guidelines exist for the investigation of sick children. Cerebrospinal fluid (CSF) should be taken from all children with suspected meningitis as well as blood cultures, when there is evidence of sepsis (temperature >38°C) but no signs to suggest localized disease. In practice this means most neonates with nonspecific signs will have both blood and CSF cultures taken before empirical antimicrobial agents are administered. Infants >1 month of age will only have a blood culture taken if no clear focus of infection, e.g., pneumonia, is evident. Guidelines exist for the use of intrapartum antimicrobial agents in febrile mothers with suspected chorioamnionitis. If prolonged rupture of membranes occurs and the neonate is admitted to the neonatal nursery, antimicrobial agents are given empirically to the infant. No record or audit information is available to assess adherence to the guidelines.

Culture of GBS from a blood or CSF sample from a QECH pediatric inpatient ≤90 days of age was the entry point to the study. Positive samples initiated a visit to the patient and the collection of clinical and, later, outcome data on the child. If a child had died with a positive GBS culture, the death was attributed to GBS. No autopsy results were available.

Most births take place at health facilities. Eighty-three percent of women who live in an urban setting will deliver at a health clinic or hospital (Malawi Demographic and Health Survey preliminary report; www.nso.malawi.net). HIV prevalence in mothers delivering at QECH was 30.2% from 2000 to 2004 (*21*)

Data Collection

Information on admissions to the neonatal nursery and pediatric ward and the number of blood cultures taken was obtained from ward admission books and laboratory records. The clinical notes of patients from whom GBS was isolated were reviewed. When no notes were available (e.g., because of death or discharge of the child before GBS was identified), the ward admission, ward round, and books containing information about patients who died on the ward were used to provide data. Information collected included date of birth, age, sex, district of residence, birthweight, and gestational age at birth (defined by maternal dates). If a child was born before 37 weeks' gestation or weighed <2.5 kg, he or she was classified as premature or of low birthweight (LBW), respectively. Age at onset of illness was used to classify the child's condition as early onset disease (EOD, defined as disease starting from birth to 6 days after birth), or late onset disease (LOD, defined as 7–90 days inclusive after birth). Outcome in hospital was recorded as dead or alive at discharge. No attempt was made to actively follow up the patients after discharge.

Clinical Definitions

Disease type was categorized by using the following criteria: 1) meningitis, pyogenic CSF from which GBS was grown; 2) probable meningitis, no GBS isolated from CSF but GBS isolated from blood and CSF findings consistent with meningitis; 3) sepsis, GBS isolated from blood with no clinical evidence of pneumonia, i.e., no increased respiratory rate or chest retraction; 4) pneumonia, GBS isolated from blood and definite clinical evidence of pneumonia, i.e., increased respiratory rate or chest retraction; 5) unknown, GBS isolated from blood but insufficient information to clinically categorize patient. The study was approved by the College of Medicine Research and Ethics Committee of the University of Malawi.

Laboratory Methods

Blood cultures are processed with a commercial blood culturing system (BacT Alert, bioMérieux, Lyons, France). CSF is processed by using standard methods. Positive blood and CSF isolates are cultured on standard media by using routine techniques. GBS was identified by its β-hemolysis on blood agar (α-hemolytic and nonhemolytic streptococci were not evaluated) and negative catalase reaction. Serogrouping was conducted by using a latex agglutination test (Pro-Lab Diagnostics, Wirral, UK). Serotyping of the GBS isolates was performed with a commercial serotyping kit according to the manufacturer's instructions (Statens Serum Institut, Copenhagen, Denmark).

Disk-diffusion antimicrobial susceptibility testing was performed according to the British Society for Antimicrobial Chemotherapy guidelines on Isosensitest agar (Oxoid Ltd, Basingstoke, UK) supplemented with 5% sheep blood media (*22*). Antimicrobial agents tested included penicillin, tetracycline, erythromycin, chloramphenicol, and ceftriaxone. All laboratory procedures were internally quality controlled. The laboratory is enrolled in the United Kingdom National External Quality Assessment Service for Microbiology.

Results

Clinical Characteristics

GBS was isolated from 57 infants in the 14-month study period; of these, 41 isolates were from blood culture only, 7 from both blood and CSF, and 9 from CSF alone. With respect to the blood cultures, 3,159 infants were admitted to the neonatal nursery during the study period; blood cultures were drawn from 681 (22%) of these patients, and 117 (17%) grew a clinically relevant isolate; 26 (22%) of these isolates were GBS. There were 4,297 children admitted to the pediatric ward; blood cultures were drawn from 1,652 (38%) of these patients, and 173 (10%) grew a clinically relevant isolate; 22 (13%) of these isolates were GBS. Admission numbers and blood cultures could not be accurately analyzed by age of the patient for the pediatric ward. Of the 57 patients, 19 died, 35 were discharged, and the outcome of 3 patients was not ascertained. The overall case-fatality rate was 33%. The Table contains a summary of the major clinical findings.

Seven (16%) of 45 infants with known gestational age were preterm, and 10 (20%) of 51 infants with known birthweight had LBW. Whether disease was early or late onset was not associated with these variables. Meningitis was more common among infants with LOD than those with EOD (Table), but the difference did not reach statistical significance ($\chi^2 = 3.4$, $p = 0.07$).

Of the isolates, 29 (51%) were from infants with EOD, and the median age of patients with initial symptoms was 1 day. The case-fatality rate was 38% for EOD. Twenty-eight isolates (49%) were from infants with LOD. The median age of LOD was 14 days (range 7–42 days), and the case-fatality rate was 29%.

Table. Characteristics of 57 case-patients with group B streptococcal infection overall and in relation to capsular serotype

		Serotype					
Clinical features	Total (%)	Ia (n = 12)	Ib (n = 3)	II (n = 4)	III (n = 32)	V (n = 1)	Unknown (n = 5)
Early onset disease*	29	7	2	3	14	1	2
Male sex†	15 (52)‡	6	2	2	4	0	1
Meningitis	5 (17)	1	1	1	2	0	0
Probable meningitis	4 (14)	1	0	0	3	0	0
Sepsis	15 (52)	5	1	1	8	0	0
Pneumonia	0 (0)	–	–	–	–	–	–
Undefined	5 (17)	0	0	1	1	1	2
Low birthweight§¶	5 (17)	2	1	0	2	0	0
Premature#**	3 (10)	2	0	0	1	0	0
Late onset disease††	28	5	1	1	18	0	3
Male sex¶	13 (46)	2	1	1	9	0	0
Meningitis	11 (39)	2	0	0	8	0	1
Probable meningitis	1 (4)	0	0	0	1	0	0
Sepsis	10 (36)	1	1	0	8	0	0
Pneumonia	3 (11)	1	0	1	1	0	0
Undefined	3 (11)	1	0	0	0	0	2
Low birthweight‡‡	5 (18)	2	0	1	1	0	1
Premature**	4 (14)	2	0	1	1	0	0
Case fatality‡							
Early onset disease	11 (38)	3	0	0	8	0	0
Late onset disease	8 (29)	3	0	0	4	0	1
Unknown	3 (5)	0	0	1	1	0	1
Sensitivity, %							
Penicillin	100	100	100	100	100	100	100
Erythromycin	79	90	100	67	78	0	78
Tetracycline	4	17	0	0	0	0	11
Ceftriaxone	100	100	100	100	100	100	100
Chloramphenicol	81	90	100	100	78	0	77

*Illness onset between birth and day 6.
†Missing information on 3 cases.
‡Expressed as a proportion of total early or late onset cases.
§Weight at birth <2.5 kg.
¶Missing information on 2 cases.
#Delivery before 37 weeks of gestation.
**Missing information on 6 cases.
††Illness onset ≥7 d after delivery.
‡‡ Missing information on 4 cases.

Serotypes

Of the 57 patients in whom GBS was identified, 52 had isolates available for serotyping. GBS were isolated from both blood and CSF in 7 cases, but both isolates were available for typing in only 4 cases; in all of these cases, the serotypes were the same. Thus, only 1 isolate per infant was included in the analysis. No GBS isolates were nontypeable. Serotype III (56%) and serotype Ia (21%) were the most frequently identified serotypes; they constituted 77% of both EOD and LOD (Figure).

Disease manifestations by serotype are shown in the Table. No discernible differences were found in EOD or LOD, clinical manifestations, or outcome by serotype. Of the 51 infants for whom a birthweight was recorded, serotype Ia caused more disease among LBW babies than among those of normal birthweight, but the trend was not significant (40% vs. 17%, respectively, $\chi^2 = 3.1$, $p = 0.08$). Disease due to serotype III was less common in those of LBW (30% vs. 68%, respectively, $\chi^2 = 4.3$, $p = 0.04$).

All GBS isolates were susceptible to penicillin, and all but 2 isolates were resistant to tetracycline (Table). Serotype and antimicrobial susceptibility were not statistically associated.

Incidence Rate Estimates

During the study period, May 1, 2004–June 30, 2005, a total of 31,458 live births were recorded in Blantyre District; a birth rate of 25.2/1,000 population. Of these births, 12,064 took place in QECH and 19,394 took place in district health centers. Therefore, the overall GBS disease incidence was 1.8/1,000 live births. The incidence of EOD was 0.92/1,000 live births, and the incidence of LOD was 0.89/1,000 live births. During the study period, 711 neonatal deaths (23% of all admissions) occurred in the neonatal nursery. A further 353 deaths (8% of all admissions) occurred in the pediatric ward, but these deaths could not be analyzed by age. GBS was implicated as a cause of death in 11 (2%) of the deaths in the neonatal nursery and in 8 (2%) of all the deaths in the pediatric ward.

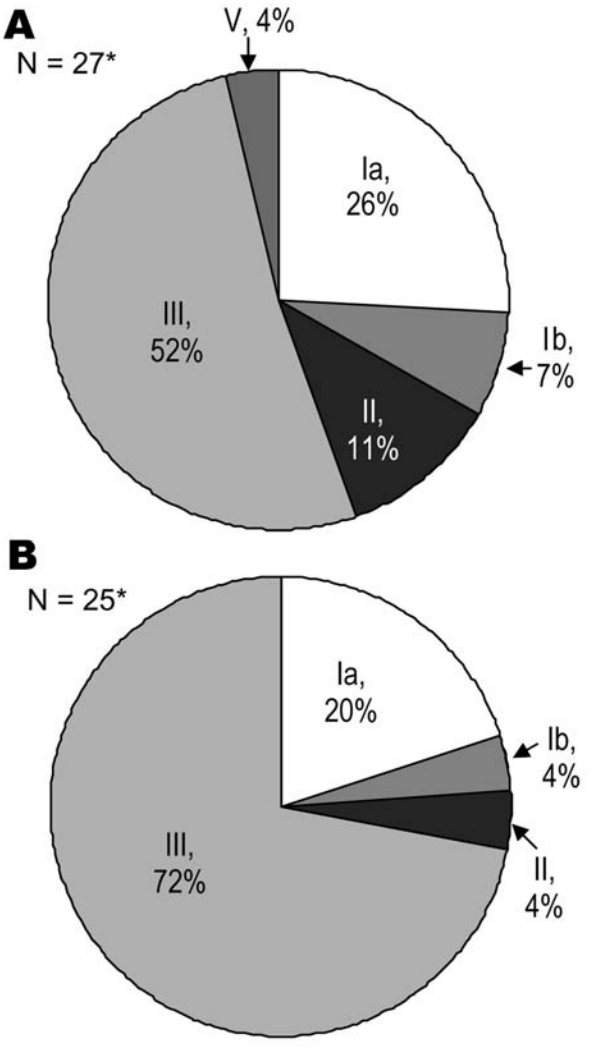

Figure. Pie chart showing serotype distribution of group B streptococcus isolates from infants with early (A) or late onset (B) disease. *Two isolates from early onset disease and 3 from late onset disease were not available for typing.

Discussion

This study adds to the growing evidence that GBS is an important cause of infectious neonatal illness and death in Africa. The incidence and outcome of disease support a more active approach for its prevention.

These results provide a benchmark for future studies with what we believe to be reasonable minimum estimates of disease incidence, despite measurement limitations in both our denominator and numerator figures. The recorded number of live births during the study period for Blantyre District is almost certainly an underestimate of the actual number. Our calculated birth rate of 25/1,000 population is low for an African urban population. A recent household demographic survey estimated the birth rate in urban Malawi at 37/1,000 (www.nso.malawi.net); thus, our live birth numbers may be underrecorded by as much as one third.

Set against this background, case-ascertainment of GBS was also suboptimal. Surveillance for GBS was passive. Only 1 in 5 infants admitted to the neonatal nursery and 2 in 5 admitted to the pediatric ward had a blood culture performed as part of the investigation of their illness. Although guidelines for assessing sick neonates exist, no audit of their implementation has been undertaken in the hospital, and shortages of syringes, needles, blood tubes, and staff are commonplace. The relatively low numbers of EOD to LOD and the high number of deaths may also be

in part explained by selective sampling of the sicker children, rather than a fundamental difference in disease pathology in Malawi.

What proportion of sick neonates was seen in QECH and how many died before they received any form of healthcare are unclear. Using data from the household demographic survey (a birth rate of 37/1,000 population and a reported neonatal death rate of 27/1,000 births [www.nso.malawi.net]) and the projected population size for Blantyre (www.nso.malawi.net/data_on_line/demography/projections/pop/bt_rural.htm and bt_city.htm), we would have expected ≈1,250 neonatal deaths in Blantyre during the study period. The 711 recorded deaths in the neonatal nursery and a proportion of the 353 deaths on the pediatric ward suggest that most neonatal deaths in Blantyre occur in QECH, but a sizeable proportion do not. We believe our results are likely to underestimate rates of GBS disease with the extent of lack of case recognition being greater than the underreporting of births.

The overall rate of GBS disease in Blantyre is higher than the overall rates of 0.6–0.9/1,000 live births reported from Western Europe (*3*,*23*,*24*). However, the rate of EOD is lower than that documented in the United States and Australia before the use of intrapartum prophylaxis, 1.7–2.0/1,000 live births, (*1*,*2*). Little information is available about rates of invasive disease in Africa for comparison. A study from the principal public-funded hospital in Johannesburg, South Africa, reported an EOD rate of 2.06/1,000 live births (*14*). That study used similar methods to our own for the rate calculations, although the calculated crude birth rate from the figures reported (≈18/1,000 population) suggests underreporting of births for the denominator and overall rates that may be similar to those in Blantyre. Another study from Johannesburg reported an EOD incidence rate of 1.16/1,000 live births (*13*) although the sociodemographic background of the population under study here is less clear. In a rural setting in East Africa, GBS bacteremia occurred at a rate of 0.66/1,000 births in neonates (*10*), which suggests that the extent of disease is greater in urban or southern Africa at this time.

The rates of EOD and LOD in this study were similar. In other settings, EOD is much more frequent than LOD when prophylaxis is not available. Our findings may in part be explained by selective sampling, but other factors may have also contributed. Some cases of EOD may have been prevented by empirical administration of antimicrobial agents, in keeping with the guidelines for chorioamnionitis and prolonged rupture of membranes, although we have no information as to the extent of this practice. Another possibility is that some of our LOD was in fact EOD because the patients had symptoms of illness for some time before seeking healthcare.

We found serotypes III and Ia to be the predominant serotypes, comprising 77% of cases; serotypes II, Ib, and V constituted the rest. This breakdown is similar to that in the single other report from Africa to date that assessed serotypes. That study, from South Africa, showed that in infants with EOD serotype III isolates caused 49.2% of disease and, together with serotype Ia isolates, caused 78.9% of disease (*14*). Studies from the industrialized world, in Finland (*25*) and Sweden (*26*), found a similar predominance of III and Ia. We found only 1 case of serotype V disease in contrast with findings from more recent studies from England (*3*), Sweden (*27*), and the United States (*28*), where serotype V is increasingly recognized as a cause of invasive disease. Serotype V was the predominant serotype, however, in a large Gambian study of maternal colonization (*8*) and was frequently identified in a similar Zimbabwean study (*29*). Neonatal disease was uncommon in the Gambian study, which suggests that factors other than bacterial serotype are required for disease to occur.

We found the rate of LOD, 0.89/1,000 live births, was slightly less than that of 1/1,000 live births reported in the South African study (*14*), although serotypes III and Ia were similarly responsible for most cases. We did not, however, define an association between serotype and timing of disease. These findings differ from reports from the industrialized world and from South Africa, where serotype III is clearly associated with LOD. This finding may also be a consequence of a case-finding bias with the youngest and sickest being more selectively investigated. The median age of patients with LOD in our study was 14 days; only 1 case occurred after the child was 28 days of age. This finding could be because hospitalized infants >28 days of age are less likely to have a blood culture taken if they have localized signs of sepsis, e.g., pneumonia. A more systematic and definitive approach to sampling will be required to further assess this finding.

Disease manifestations were similar to those in other studies, apart from a higher proportion of EOD (31%) manifesting as meningitis. Other studies have reported 6%–10% of EOD as meningitis (*2*,*3*). The high rate could be explained by preferential sampling of the sickest infants in circumstances of limited resources. We found that reliably differentiating sepsis from pneumonia was problematic, again, as a result of the lack of investigative facilities; thus, we may have underdiagnosed cases of pneumonia.

The case-fatality rate in this case-series resembles that seen in the United States in the 1970s, when the case-fatality rate was >50% (*30*,*31*). Our case-fatality rate is much higher than that more recently recorded in Europe (8%–9%) (*3*,*25*,*32*), the United States (4%–6%) (*2*), or South Africa (19.8% for EOD and 13.6% for LOD) (*14*). This finding likely reflects the difficulties of managing

these infants with limited resources, lack of intensive care facilities, and late seeking of healthcare for some infants, and possibly coexistent illness such as HIV.

We do not have any information on HIV status of mothers or children in our study. Speculation that the emergence of GBS as a pathogen in southern and eastern Africa is related to HIV infection is tempting. HIV-infected adults have defects in the humoral immune responses to polysaccharide antigens, best recognized in the case of pneumococci (*33*). GBS capsular polysaccharides are similar to pneumococcal capsular polysaccharides, and serologic cross-reactivity is recognized (*34*). Thus, HIV-infected women might carry more GBS and might transfer less transplacental protection. Further research in this area is required.

We found all isolates were susceptible to the β- lactam antimicrobial drugs and that most (96%) were resistant to tetracycline, as would be expected. However, 21% of isolates were resistant to erythromycin, which is a higher proportion than that reported from the United Kingdom (4% erythromycin resistant) (*3*) but similar to that reported from France (21.4%) (*35*), the United States (20%) (*36*), and Zimbabwe (14%) (*37*). Chemoprophylaxis with antenatal azithromycin is under evaluation as a means to improve pregnancy outcome in Malawi, primarily by reducing chorioamnionitis (including that caused by GBS) and possibly malaria. Were this treatment to become available, this higher rate of resistance to macrolides may limit the value of this approach in reducing GBS-associated pathology and could limit options for intrapartum antimicrobial prophylaxis for penicillin-allergic patients.

From our data, interventions to prevent GBS disease appear warranted. Chemoprophylaxis has been successful in reducing rates of EOD in many countries (*2*,*5*). An intrapartum screening–based approach for prophylaxis would not be feasible because microbiology facilities are lacking in both QECH and the surrounding districts. Risk-based prophylaxis could be considered. However, only a small proportion of these infants were of LBW (10), and of these only 7 were noted to be premature. We had insufficient information about the obstetric histories to examine risk factors such as prolonged rupture of membranes, maternal fever, and prolonged labor. Vaginal disinfection with microbicides during labor has been considered in developing countries (*38*). In Malawi, the use of chlorhexidine wipes significantly reduced neonatal and maternal sepsis–related illness and death at QECH in a study in which the primary aim was to reduce perinatal HIV transmission (*39*). This approach is likely to be less effective when a high proportion of deliveries take place without healthcare supervision, and this fact may in part explain the failure of this technique to become routine practice.

A vaccine-based strategy would be particularly suited for use in the developing world, where maternal immunization with tetanus toxoid is a safe and valuable part of routine antenatal care (*40*). However, the impetus to develop these vaccines has diminished because of the success of chemoprophylaxis in industrialized countries. Vaccination would appear to offer the widest coverage for a successful intervention and would likely offer protection from both EOD and LOD. Our study suggests that an efficacious 2-valent vaccine aimed at serotypes Ia and III could prevent >75% of invasive disease due to GBS in Malawian infants.

In summary, we have demonstrated a pattern of neonatal GBS disease similar in scale and serotype distribution to reports from the industrialized world but with a significantly worse outcome. We suggest that the effectiveness of vaginal disinfection should be further assessed and that the currently stalled vaccine development programs of recent years be restarted with a clear intention of assessing their role in the developing world.

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Dr Gray is a clinical microbiologist based at the Malawi-Liverpool–Wellcome Trust Research Laboratories in Blantyre, Malawi. Her interests are in bacterial and fungal disease in African populations.

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Address for correspondence: Katherine J. Gray, Malawi-Liverpool–Wellcome Trust Laboratories, PO Box 30096, Blantyre, Malawi; email: kgray@africa-online.net

Deaths from Cysticercosis, United States

Frank J. Sorvillo,*† Christopher DeGiorgio,* and Stephen H. Waterman‡

Cysticercosis has emerged as a cause of severe neurologic disease in the United States. We evaluated cysticercosis-related deaths in the United States for 1990–2002 by race, sex, age, state of residence, country of birth, and year of death. A total of 221 cysticercosis deaths were identified. Mortality rates were highest for Latinos (adjusted rate ratio [ARR] 94.5, relative to whites) and men (ARR = 1.8). The mean age at death was 40.5 years (range 2–88). Most patients (187 [84.6%]) were foreign born, and 137 (62%) had emigrated from Mexico. The 33 US-born persons who died of cysticercosis represented 15% of all cysticercosis-related deaths. The cysticercosis mortality rate was highest in California, which accounted for ≈60% of all deaths. Although uncommon, cysticercosis is a cause of premature death in the United States. Fatal cysticercosis affected mainly immigrants from Mexico and other Latin American countries; however, US-born persons were also affected.

Cysticercosis, a parasitic infection caused by the larval form of the pork tapeworm, *Taenia solium*, has been increasingly recognized as a cause of severe but preventable neurologic disease in the United States (*1*–*5*). Reports documenting hundreds of cases, mainly of neurocysticercosis, have drawn attention to this previously underrecognized disease (*6*,*7*).

Cysticercosis has a complex life cycle. The larval infection, cysticercosis, is transmitted through the fecal-oral route. Eggs from the adult tapeworm *T. solium*, which are directly infectious, are shed in the feces of a human tapeworm carrier and subsequently ingested by pigs, the usual intermediate host (*8*). The oncosphere embryos emerge from the eggs, penetrate the intestinal wall, and are disseminated by the bloodstream to various tissues where the larval stage, or cysticercus, develops. The cycle is completed when humans, the only naturally infected definitive host, consume raw or undercooked pork containing cysticerci, which attach to the small bowel and develop into the adult tapeworm. However, humans may also become infected with the larval stage when eggs are ingested, typically in contaminated food or water. Neurocysticercosis, the most severe form of the disease, occurs when larvae invade tissue of the central nervous system.

Cysticercosis in the United States affects mainly immigrants from Latin America, where the disease is endemic. However, cysticercosis acquired in the United States has been repeatedly documented over the past 15 years, and travel-related infection in US-born persons has been reported (*9*–*11*). Given the ongoing sizeable immigration from disease-endemic areas, cysticercosis will grow in clinical and public health importance; however, data on cysticercosis in the United States are lacking. The disease is not nationally reportable, few local jurisdictions require reporting, and surveillance systems for cysticercosis have rarely been implemented (*10*,*12*). In the absence of effective surveillance, the true prevalence of cysticercosis in the United States is largely unknown. Although several hospital-based series have provided valuable insights into the occurrence of cysticercosis, they reflect only a portion of actual cases and do not measure the true effect of the disease on the general population and at-risk populations. Moreover, few data exist on cysticercosis as a cause of death in the United States (*6*,*13*). To augment current information on the effect of cysticercosis in the United States, we evaluated national mortality records for cysticercosis-related deaths for the 13-year period 1990–2002.

Methods

Data Source

Mortality data were obtained from the National Center for Health Statistics (NCHS). Death certificates, which are required by state law, must indicate a cause or sequence of

*University of California, Los Angeles, California, USA; †Department of Public Health, Los Angeles County, California, USA; and ‡University of California, San Diego, California, USA

events that led to death, as determined by the attending physician. If a physician is not in attendance or the death is accidental or occurs under suspicious circumstances, then cause of death is determined by the local coroner or medical examiner. Death certificate data are transmitted from state jurisdictions to NCHS. The US Multiple Cause of Death Files for 1990 through 2002 were searched for listings of cysticercosis (ICD-9 code 123.1 for 1989–1998 and ICD-10 code B69 for 1999–2002). Availability of this national data source typically has a 3-year lag time. The multiple cause of death data contain all causes of death provided by the physician or coroner. Such information is more complete than data files with primary cause of death only. Additional variables extracted from the death record included age, sex, race/ethnicity, level of education, country of birth, place of death, date of death, and other concurrent conditions.

Data Analysis

Cysticercosis mortality rates per million population were calculated. Population data were obtained from the US Census Bureau. Crude cysticercosis mortality rates and 95% confidence intervals (CIs) were computed by age group (<1, 1–4, 5–14, 15–24, 25–34, 35–44, 45–54, 55–64, 65–74, 75–84, ≥85 years), sex, race/ethnicity (white, black, Latino, Asian, Native American), and state of residence. Age-adjusted rates were calculated for race/ethnicity, sex, and state. The US population for the year 2000 was used as the standard population for all age-adjusted rates. Rate ratios, adjusted rate ratios, and 95% CIs were also computed. Demographic characteristics of US-born patients were compared with those of foreign-born patients. The χ^2, Fisher exact, and Student *t* tests were used where appropriate to assess apparent differences. Conditions occurring with cysticercosis were examined and compared with a random sample of deaths from causes other than cysticercosis matched by patient age, sex, and race/ethnicity. Matched odds ratios and 95% CIs were calculated for each condition.

Results

Over the 13-year study period, 221 cysticercosis deaths were identified, representing an annual age-adjusted mortality rate of 0.06 per million population (95% CI, 0.05–0.07). Most persons who died from cysticercosis (187 [84.6%]) were Latino; 15 (6.8%) were white, 13 (5.9%) were black, 5 (2.3%) were Asian, and 1 (0.5%) was Native American (Table 1). By sex, 137 (62.0%) were male, and 84 (38.0%) were female. Mean age at death was 40.5 years (range 2–88 years). Most persons who died (187 [84.6%]) were foreign born, and 137 (62%) of all persons who died had emigrated from Mexico. Ten (77%) of the black and all 5 of the Asian persons who died were foreign born. At least 1 cysticercosis death was reported from 20 states; California accounted for 57% (126 deaths), and Los Angeles County, California, recorded 32% (70 deaths) of the US total (Figure). Cysticercosis was listed as the primary cause of death for 165 (74.7%) persons.

Table 1. Demographic characteristics of 221 patients with fatal cysticercosis, United States, 1990–2002

Characteristic	No.	%
Sex		
Male	137	62.0
Female	84	38.0
Race/ethnicity		
White	15	6.8
Black	13	5.9
Latino	187	84.6
Asian/Pacific Islander	5	2.3
Native American	1	0.5
Age group, y		
1–4	1	0.5
5–14	5	2.3
15–24	37	16.7
25–34	66	29.9
35–44	29	13.1
45–54	36	16.3
55–64	20	9.1
65–74	15	6.8
75–84	9	4.1
≥85	3	1.4
Education, y*		
<12	135	61.1
12	43	19.5
>12	25	11.3
Country of birth†		
United States	33	14.9
Mexico	137	62.0
Other	50	22.6

*Unknown for 18 persons.
†Unknown for 1 person.

Age-adjusted cysticercosis mortality rates were highest for Latinos (adjusted rate ratio [ARR] = 94.5, 95% CI 56.9–156.9, relative to whites) and men (ARR = 1.8, 95% CI 1.4–2.3) (Table 2). The mean age at death was 40.5 years; >60% of deaths occurred in persons <45 years of age. Most persons (61%) had <12 years of education. Although no clear temporal trend was noted, cysticercosis deaths varied by year; most deaths (23) occurred in 1992 and 1997.

The 33 cysticercosis deaths in US-born persons represented 15% of all cysticercosis deaths. Ten (30.3%) of these 33 persons were California residents. US-born persons with fatal cysticercosis had higher educational levels ($p = 0.02$) and were older (mean age 50.1 vs. 38.7 years, $p<0.01$) than foreign-born persons with fatal cysticercosis (Table 3). Although 52% of US-born persons who died were Latino, this proportion was lower than that for foreign-born persons (90.4%, $p<0.01$). At least 1 death of a

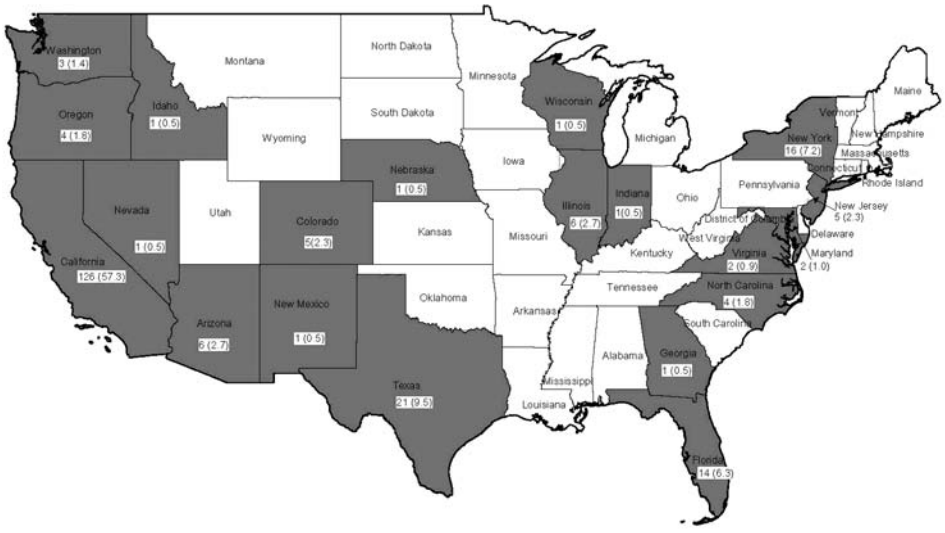

Figure. Frequency and percentage of fatal cysticercosis cases by state, United States, 1990–2002. Shaded areas indicate states with deaths from cysticercosis.

US-born person was reported in each year of the study period.

Principal concurrent conditions listed as contributing to death included hydrocephalus in 58 (26.2%) persons, cerebral edema in 23 (10.4%), cerebral compression in 16 (7.2%), and epilepsy/convulsions in 12 (5.4%). These conditions were significantly more common in persons who died of cysticercosis than in matched controls ($p<0.001$). Septicemia was recorded for 15 (6.8%) of persons with fatal cysticercosis, but this figure was not significant. Reported place of death included inpatient facility (64.7%), emergency room or outpatient clinic (9.5%), nursing home (9.5%), and residence (11.3%).

Discussion

Our findings indicate that in the United States, cysticercosis is a cause of premature death, particularly among young Latinos, and may be a more frequent cause of death than previously recognized. Substantially more deaths occurred in California, particularly Los Angeles County, and in southwestern states bordering Mexico. Although cysticercosis causes death mainly among Hispanic immigrants, our findings indicate that this larval tapeworm causes infection and death in US-born persons as well.

The elevated cysticercosis mortality rates for Latinos reflect the substantial immigration from *T. solium*–endemic areas of Mexico and other Latin American countries. Over 70% of cysticercosis deaths were of persons born in Mexico. Legal immigration to the United States from Mexico during 1991–2000 was >2.2 million; >1 million additional immigrants came from Central and South American countries (*14*). Moreover, undocumented immigration from such areas continues to occur in considerable numbers. The US Immigration and Naturalization Service estimates that 7 million unauthorized immigrants (4.8 million of these from Mexico) were residing in the United States in January 2000 and that an average of 350,000 immigrate each year (*14*). Cysticercosis and taeniasis are widely prevalent in many Latin American countries. Autopsy studies conducted in Mexico have reported cysticercosis prevalence from 2.8% to 3.6%, and serosurveys have demonstrated infection rates of ≥20% in some areas of Peru, Guatemala, and Bolivia (*3*,*15*). A recent study of farm workers in southern California documented seroprevalence of 1.8% for cysticercosis and 1.1% for taeniasis, comparable to that in cysticercosis-endemic areas (*16*).

We noted several cysticercosis deaths of persons who were born in the United States, which indicates the possibility of locally acquired disease. Transmission of cysticercosis in the United States has been repeatedly documented over the past 20 years and can often be traced to the presence of a tapeworm carrier among household members or other close personal contacts (*3*,*9*–*11*,*17*). An outbreak of neurocysticercosis in an Orthodox Jewish community in New York City implicated domestic employees from Latin

Table 2. Cysticercosis mortality rates, United States, 1990–2002

	Rate/10^6 population (95% CI)	ARR (95% CI)*
Sex		
Male	0.08 (0.07–0.1)	1.8 (1.4–2.3)
Female	0.05 (0.04–0.06)	Referent
Race/ethnicity		
White	0.006 (0.003–0.008)	Referent
Black	0.03 (0.01–0.05)	5.1 (3.1–8.6)
Latino	0.56 (0.47–0.65)	94.5 (56.9–156.9)
Asian/Pacific Islander	0.04 (0.0–0.07)	6.7 (4.0–11.2)
Native American	0.04 (0.0–0.12)	6.2 (3.7–10.3)

*CI, confidence interval; ARR, age-adjusted rate ratio.

Table 3. Selected characteristics of US-born and foreign-born persons with fatal cysticercosis, United States, 1990–2002

Characteristic	US-born, n = 33, no. (%)	Foreign-born, n = 187, no. (%)	p value
Sex			
Male	22 (66.7)	114 (61.0)	0.53
Female	11 (33.3)	73 (39.0)	
Race/ethnicity			
White	12 (36.4)	3 (1.6)	<0.001
Latino	17 (51.5)	169 (90.4)	
Black	3 (9.1)	10 (5.4)	
Asian/ Pacific Islander	0 (0)	5 (2.7)	
Native American	1 (3.0)	0 (0)	
Mean age, range	50.1, 2–88	38.7, 7–86	<0.01
Education, y			
<12	12 (36.4)	123 (65.8)	<0.001
12	10 (30.3)	33 (17.7)	
>12	8 (24.2)	17 (9.1)	
Unknown	3 (9.1)	14 (7.5)	

America who harbored *Taenia* infections as the probable source of infection (*9*). A pilot surveillance system implemented in Los Angeles County during 1988–1990 identified 10 locally acquired cases among 138 cases reported and found a tapeworm carrier among household contacts for 5 (7%) of 72 overall cases investigated (*10*).

Alternatively, the occurrence of cysticercosis among US-born persons may reflect travel-related exposure and infection. Travel-associated cysticercosis, mainly in persons who have visited Mexico and other Latin American countries, has been previously documented; however the risk and frequency of such infections are unknown (*10*,*18*). The Los Angeles County surveillance system identified 9 probable travel-related cases, which represented 6.5% of the total cysticercosis cases. In a study of cysticercosis in Texas, de La Garza and colleagues reported 6 cases in US-born persons, all of whom had a history of frequent travel to rural Mexico or Central America (*19*). Substantial numbers of US residents travel to cysticercosis-endemic areas each year and may be exposed to food and water contaminated with *T. solium* eggs. Therefore, many of the US-born persons likely acquired infection during travel to endemic areas. Food and water precautions for travelers to cysticercosis-endemic regions should be reinforced.

Although 21 states had at least 1 death from cysticercosis, mortality rates were highest in California and other border states. Cysticercosis deaths were also routinely recorded in New York and Florida. This observed geographic focus of cysticercosis deaths reflects immigration patterns in states that include substantial populations of immigrants from cysticercosis-endemic areas, particularly Mexico and other areas of Latin America.

The sex disparity noted in this study is consistent with data from our recent population study, which demonstrated a significantly higher prevalence of cysticercosis in men (*16*) and likely reflects the greater immigration of young men in search of employment. Such immigration patterns may also explain the relatively young age observed; >60% of cysticercosis deaths in our study were in persons <45 years of age, a heavy toll among young, highly productive persons.

Although we could not assess whether problems with access to healthcare contributed to cysticercosis deaths, >20% of deaths occurred at home, in an emergency room, or in an outpatient setting. Reduced access may have an effect on cysticercosis deaths; additional data on this issue would be useful.

Several large facility-based case series studies have reported that the number of deaths from cysticercosis is relatively low and that the case-fatality rate is <1%. However, such facility-based studies, although providing valuable information, have substantial limitations and may underestimate cysticercosis as a cause of death. Limited data from the pilot Los Angles County surveillance system found a mortality rate of ≈6% (8 of 138 incident cases), and the Dixon study of British troops who had served in India reported mortality rates of nearly 10% (*10*,*20*). However, these case-fatality rates must be viewed with caution because they may reflect underdiagnosis or underreporting of less severe cases and therefore probably represent overestimates. Mortality rates have been reported to be higher for surgically treated patients and those with hydrocephalus, primarily because of increased intracranial pressure and shunt-related infection (*21*). We found that hydrocephalus, cerebral compression/edema, and epilepsy/convulsions were common concurrent conditions recorded on the death certificate. Fatal cysticercosis may also occur in persons who have ingested large numbers of eggs, which may cause an overwhelming, fatal acute infection with numerous larvae and severe central nervous system pathologic changes. Racemose cysticercosis, a phenomenon in which cysticerci continue to grow and proliferate through tissue, may also have a poor prognosis. Newer, less invasive, endoscopic surgical techniques for removing intraventricular cysticerci offer promise of reducing mortality rates (*22*).

Our data, although population based, likely underestimate cysticercosis deaths for several reasons. To be listed on the death certificate, cysticercosis must be recognized and diagnosed, which requires confirmation of infection through biopsy, autopsy, or specialized serologic testing (*23*). Consequently, some cases of fatal cysticercosis likely go undiagnosed and unrecognized, which would result in the miscoding of cysticercosis-related deaths as other conditions. For this reason, death records may be biased and likely underestimate deaths from cysticercosis. The absence of fatal cases reported from Kansas, despite a

recent report documenting widespread cysticercosis (*24*), appears to support the notion of underrecognition of fatal cases and suggests caution in interpreting geographic distribution. Our findings demonstrate the benefits of using multiple-cause-of-death data instead of the traditional underlying-cause-of-death data alone for estimating deaths from cysticercosis. An additional 56 (25.3%) cases were identified by using multiple-cause–coded files.

The use of death certificates to assess the effect of disease has advantages and limitations. Because submission of death certificates is required by state law, ascertainment and registration of deaths are virtually complete. Use of mortality records therefore provides population-based data and avoids the potential biases of facility-based data or other data that are not population based. Mortality data can also indicate disease severity and contribute to measures of disease load. However, data from death certificates have several limitations, including the possible coding of inaccurate information through careless completion of cause of death, coding errors, and misclassification of variables such as race/ethnicity (*25*,*26*). Reporting of country of birth may also be inaccurate, and persons with cysticercosis who are recorded as having been born in the United States may, in fact, be foreign born. Deaths from cysticercosis represent only a small fraction of total disease burden. In addition, census data and intercensus population estimates used for the calculation of rates may be uncertain. For these reasons, our estimate of cysticercosis mortality rate must be interpreted with caution.

Cysticercosis can cause severe neurologic disease and death and result in substantial cost to the healthcare system, yet simple public health measures can reduce or eliminate this parasitic disease. In fact, cysticercosis has been identified as 1 of 6 potentially eradicable diseases (*27*). Because most cysticercosis cases in the United States are imported, efforts to control the disease in cysticercosis-endemic regions will reduce disease in the United States. Such control activities can also reduce the likelihood of travel-related infection. State and local health authorities in affected areas of the United States should consider implementing surveillance and follow-up of cysticercosis patients, including attempts to identify and treat tapeworm carriers among household members and other close personal contacts. The availability of a sensitive and specific test for *T. solium* infection that can be performed from blood samples obtained through simple finger stick will facilitate such follow-up (*28*). Given the importance of cysticercosis in border areas, collaborative studies with Mexican public health authorities on the prevalence and incidence of cysticercosis in the border regions should be implemented (*29*,*30*).

Dr Sorvillo is professor in-residence in the Department of Epidemiology at the School of Public Health, University of California, Los Angeles, California. His research interests include the epidemiology and control of infectious diseases, particularly parasitic agents.

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Address for correspondence: Frank J. Sorvillo, Department of Epidemiology, School of Public Health, UCLA, Box 951772, Los Angeles, CA 90095, USA; email: fsorvill@ucla.edu

Community-associated Methicillin-resistant *Staphylococcus aureus* Isolates Causing Healthcare-associated Infections[1]

Cynthia L. Maree,* Robert S. Daum,† Susan Boyle-Vavra,† Kelli Matayoshi,‡[2] and Loren G. Miller*‡

We noted a marked increase in healthcare-associated (HA) methicillin-resistant *Staphylococcus aureus* (MRSA) infections caused by isolates phenotypically consistent with community-associated (CA)-MRSA strains. To study this trend, we retrospectively examined all HA-MRSA isolates from patients in our institution during 1999–2004. An isolate was considered an SCC*mec*IV phenotype if it had antimicrobial drug susceptibilities consistent with typical CA-MRSA isolates. Our phenotypic definition was validated in a limited subset of isolates by SCC*mec* genotype, pulsed-field gel electrophoresis, and multilocus sequence typing. Among 352 patients with HA-MRSA isolates, SCC*mec*IV phenotype increased from 17% in 1999 to 56% in 2003 (p<0.0001). Antimicrobial drug-susceptibility phenotype and genotype were consistent in 21 (91%) of 23 isolates. In a multivariate model, the SCC*mec* type IV phenotype was independently associated with wound culture source, later year of collection, and MRSA isolated earlier during hospitalization. In conclusion, MRSA isolates phenotypically similar to CA strains have become the predominant isolates associated with HA-MRSA in our hospital.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most frequently identified antimicrobial drug–resistant pathogen in US hospitals (*1*). The epidemiology of infections caused by MRSA is rapidly changing. In the past 10 years, infections caused by this organism have emerged in the community. The 2 MRSA clones in the United States most closely associated with community outbreaks, USA400 (MW2 strain, ST1 lineage) and USA300, often contain *pvl* genes and, more frequently, have been associated with skin and soft tissue infections (*2*,*3*). Outbreaks of community-associated (CA)–MRSA infections have been reported in correctional facilities, among athletic teams, among military recruits, in newborn nurseries, and among men who have sex with men (*4*–*7*). CA-MRSA infections now appear to be endemic in many urban regions and cause most CA–*S. aureus* infections (*5*,*6*,*8*–*10*).

CA-MRSA isolates were first recognized by distinct resistance profiles of antimicrobial drugs that lacked resistance to older antimicrobial drugs (*11*–*13*). Several groups have noted these distinct susceptibility patterns appearing in isolates from hospitalized patients. Denis et al. noted that since 1995, MRSA isolates in Belgian hospitals were losing resistance to older antimicrobial drugs such as gentamicin and clindamycin (*14*). A Spanish hospital experienced a decrease in gentamicin-resistant MRSA isolates (from 97% in 1998 to 20% in 2002) and a simultaneous increase in MRSA isolates carrying the SCC*mec* type IV cassette (from 0% prevalence in 2000 to 23% prevalence in 2002) (*15*). A French group noted a similar finding in their hospitals over an 11-year period and found a correlation between isolates that contained SCC*mec* type IV and susceptibility profiles to ≥3 antimicrobial drugs (*16*). However, these investigations did not

*David Geffen School of Medicine at the University of California, Los Angeles, California, USA; †University of Chicago, Chicago, Illinois, USA; and ‡Los Angeles Biomedical Institute at Harbor-UCLA Medical Center, Torrance, California, USA

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[2]Current affiliation: University of Southern California School of Pharmacy, Los Angeles, California

distinguish between cultures obtained from patients hospitalized with CA infection and those with hospital-associated (HA) infections. Thus, it is unclear whether these trends in decreased antimicrobial drug resistance and increased number of MRSA isolates that contained SCC*mec* type IV were due to increased hospitalization of patients with CA-MRSA infections or to an increased prevalence of isolates containing SCC*mec* type IV among HA-MRSA isolates.

Some MRSA strains associated with CA infection have been noted to cause HA infections. Outbreaks of HA infections caused by isolates containing SCC*mec* type IV have been reported from Australia and the United States. Affected populations have included postpartum women and patients undergoing prosthetic joint replacement (*17–19*). Another recent report demonstrated that CA strains had emerged as a substantial cause of HA bloodstream infections (*20*). However, these reports are anecdotal, and data examining temporal trends are lacking.

At our institution, which is located in an area in which CA-MRSA infections are endemic, we have noted a large increase in HA infections caused by MRSA isolates that, by assessment of antibiotic susceptibility patterns, appear to carry the SCC*mec* type IV element (e.g., susceptible to gentamicin, clindamycin, and trimethoprim-sulfamethoxazole) (*6*,*10*,*21*). The aim of this study was to quantify this trend over a 6-year period.

Methods

Population

To find patients with HA-MRSA infections, we identified all cultures obtained ≥72 hours after hospitalization that grew MRSA, from January 1, 1999, through December 31, 2004, at Harbor-UCLA Medical Center, a tertiary-care, urban, county hospital in Los Angeles County. At this hospital, surveillance cultures for MRSA colonization are not routinely performed; therefore, cultures positive for MRSA are likely to reflect infection rather than colonization. For a given patient, we examined only data from the first positive culture and excluded patients who had positive cultures both ≥72 hours and <72 hours after admission. If a patient had been hospitalized more than once during the study period, only data from the first hospitalization were retained. A standardized instrument was used to abstract data from the medical record of each patient. Information obtained included demographics, admission date and time, hospital location, antimicrobial drug susceptibility of the MRSA isolate, and time, date, and source of the MRSA culture.

We obtained only MRSA blood isolates for molecular typing because the clinical microbiology laboratory discards all other types of isolates after identification is complete. In vitro susceptibilities were reported as minimal inhibitory concentrations and performed with the VITEK system (bioMérieux, Durham, NC, USA), according to the protocols of the Clinical and Laboratory Standards Institute (CLSI). The investigation protocol was reviewed and approved by the Institutional Review Board of Harbor-UCLA Medical Center.

Molecular Characterization of Strains

Molecular typing was performed at the University of Chicago by investigators who were blinded to the clinical details and antibiograms of the isolates.

SCC*mec* Typing

PCR was performed to detect *mecA* by using the primer pair mecAF/mecAR (*22*). SCC*mec* elements were distinguished by the molecular architecture of the *ccr* and *mecA* complexes (*21*,*23*,*24*). PCR typing of SCC*mec* types I–IV was performed under the conditions previously described (*24*,*25*). SCC*mec* type II (*ccrAB* complex type 2 and *mec* complex class A), SCC*mec* type III (*ccrAB* complex type 3 and *mec* complex class A), and SCC*mec* type IV (*ccrAB* complex type 2 and *mec* complex class B) were assigned according to published criteria (*25*). PCR primers used to detect *mecI* (primers mI3/mI4), the *mecR1* membrane spanning region (MS) (primers mcR3/mcR4), and the *mecR1* penicillin-binding region (PB) (primers mcR1/mcR5) were originally reported by Suzuki et al. (*26*). Screening for *ccrAB* complex types 1, 2, and 3 (*ccrAB* 1, 2 and 3) was accomplished with a multiplex PCR assay that uses a mixture of 4 primers designed by Ito et al., consisting of a common forward primer (β2) and reverse primers, α2, α3, and α4 specific for *ccrAB* complexes 1, 2, and 3. Thermocycler conditions used have been described (*27*). The presence of the *ccrAB* gene complex allotype 4 (*ccr complex 4*) was assessed in a separate reaction that used the primer pair ccrA4F and ccrB4R (*27*). Screening for the *ccrC* gene (*ccr complex 5*) was performed with a forward primer (γF) in combination with the reverse primer γR described by Ito et al. (*28*). Prototype strains used for SCC*mec* typing were NCTC10442 (SCC*mec* I), N315 (SCC*mec* II), 85/2082 (SCC*mec* III), MW2 (SCC*mec* IV), and WIS (SCC*mec* V). The control strain used for detection of *ccrAB4* was *S. epidermidis* strain ATCC 12228, which contains *ccrAB4* in the non–*mec*-containing SCCcomposite island (*24*).

MLST

MLST was performed by PCR amplification and sequencing of 7 housekeeping genes by using the primer pairs designed by Enright et al (*29*). Denville Taq-Pro Complete (Denville Scientific, Metuchen, NJ, USA) or the Taq DNA Polymerase (Promega, Madison, WI, USA) was used for the PCR reactions. PCR products were evaluated

on an agarose gel and purified by using Millipore 96-well Montage (Billerica, MA, USA) plates according to manufacturer's instructions. The purified templates were sequenced at the University of Chicago Core Sequencing Facility and evaluated with the use of Vector NTI software (Invitrogen, Carlsbad, CA, USA). Each sequence was submitted to the MLST database website (www.mlst.net) for assignment of the allelic profile and sequence type (ST).

Screening for *pvl* Genes

Isolates were screened for the *lukF-PV* and *lukS-PV* genes encoding the components of the PVL toxin by PCR amplification of a 433-bp product that includes a portion of both the *lukS-PV* and *lukF-PV* ORFs by using the primer pair luk-PV-1/ luk-PV-2 (final concentration 0.2 μM) designed by Lina et al. (*30*). The thermocycler conditions have been described (*27*).

Case Definition and Data Analysis

A standardized definition of CA-MRSA infection was created by the Centers for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance sites (*31*). Using this definition, we defined HA-MRSA infections as those MRSA infections that did not meet the definition of CA-MRSA infections. Specifically, we defined an MRSA isolate as HA associated if the original entry criteria of hospitalization for ≥72 hours before culture acquisition was met and if in the year before the present hospitalization, the patient had had any 1 of the following: hospitalization, surgery, residency in a long-term care facility, and hemodialysis or peritoneal dialysis, or at the present admission had indwelling percutaneous devices or catheters. A CA infection was defined as a culture-confirmed MRSA infection without any of the above criteria. However, if the patient did not meet any of the above criteria, had an infection at the time of admission, and the culture of the infection on admission was taken ≥72 hours after admission, then the infection was considered CA. An example of this situation would be a deep tissue infection microbiologically diagnosed from a surgical biopsy specimen 4 days after the patient's admission.

To validate our definition of HA-associated infection, we reviewed 105 (30%) randomly selected charts of the patients with MRSA infections identified ≥72 hours after hospitalization. The purpose of this validation was to confirm that these cultures did not reflect CA infections that were diagnosed late (>72 hours) in the hospital course. Of note, in the CDC definition, an infection is considered HA if it occurs >48 hours after admission. Yet, we chose ≥72 hours as a cut-off to more conservatively capture HA infections, i.e., to minimize the miscategorization of CA infections as HA infections.

We then defined MRSA strains as having the SCC*mec* type IV phenotype if the isolates were resistant to oxacillin and susceptible to gentamicin, clindamycin, and trimethoprim-sulfamethoxazole. All other isolates were considered to be phenotypically non–SCC*mec* type IV.

Characteristics were compared between patients infected with the non–SCC*mec* type IV phenotype isolates and those infected with SCC*mec* type IV phenotype isolates by using a χ^2 or *t* test, as appropriate. No adjustments were made for multiple comparisons. Temporal trends in the proportion of the SCC*mec* type IV phenotype were compared with the Cochran-Armitage test of trends. A multivariate analysis that predicted phenotypically SCC*mec* type IV isolates was performed by using an unconditional logistic regression model and a backward model selection method. A p value of ≤0.05 was defined as statistically significant. Data analysis was done with SAS software version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Population Characteristics

We identified 352 patients who had HA-MRSA cultures; 229 (65%) were men, and the median age was 50 years (mean 49.5 years). In the subset of medical records reviewed for validation of HA or CA status, none of the patients' infections (0/105) fit our definition of a CA infection. The SCC*mec* type IV phenotype was identified in 128 (36%) of these 352 patients. Compared with the non–SCC*mec* type IV phenotype, patients with the SCC*mec* type IV phenotype were younger (median age 48 vs. 54 years, p = 0.02) and had the defining culture taken earlier in the hospitalization (median 8 vs. 15 days, p = 0.01). Finding an isolate with the SCC*mec* type IV phenotype was more likely if the culture source was from a wound, blood, or source other than sputum (odds ratio [OR] 2.9, 95% confidence interval [CI] 1.7–5.0, p<0.0001; OR 2.6, 95% CI 1.2–5.7, p = 0.02; and OR 1.2, 95% CI 0.6–2.3, p = 0.69) (Table 1).

Validation of the SCC*mec* Phenotype Definition

Of the 352 cultures, 35 were recovered from blood and were potentially available for genetic analysis. We were able to subculture 24 of the blood isolates. We could not perform SCC*mec* typing on 1 of the 24 growing isolates. The 23 remaining isolates were representative of each year of the 6-year period except 1999, when no isolates could be recovered.

Twelve isolates carried the SCC*mec* type IV element, and 9 also carried the *pvl* genes (Table 2). Eleven isolates carried the SCC*mec* type II element; none carried *pvl*. The clinical definition of the SCC*mec* IV phenotype was fulfilled by 11 (92%) of the 12 isolates that carried the

Table 1. Patients with healthcare-associated MRSA isolates, 1999–2004, and predictors of SCC*mec* type IV phenotype*

Characteristic	Patients with HA-MRSA, isolates, % (no.)	SCC*mec* phenotype,% (no.)		Predictors of SCC*mec* type IV phenotype			
				Bivariate anaylsis		Multivariate analysis	
		Type IV	Type II/III	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Total	(352)	36 (128)	64 (224)				
Sex							
F	35 (123)	36 (44)	64 (79)		0.87		>0.05
M	65 (229)	37 (84)	63 (145)				
Age, y							
Mean	50 ± 19 SD	47 ± 1.7 SE	51 ± 1.3 SE		**0.02**		>0.05
Median (range)	50 (<1–97)	48 (<1–87)	54 (<1–97)				
Time from admission to specimen collection, d							
Mean	19 ± 21 SD	15 ± 1.9 SE	21 ± 1.4 SE		**0.01**	0.88 (0.8–0.98)†	**0.02**
Median (range)	12 (4–184)	8 (4–174)	15 (4–184)				
Culture specimen source							
Blood	10 (35)	46 (16)	54 (19)	2.6 (1.2–5.7)	**0.02**	2.2 (0.97–5.0)	0.058
Sputum	34 (188)	25 (29)	75 (89)	Reference		Reference	
Wound	38 (133)	49 (65)	51 (68)	2.9 (1.7–5.0)	**<0.0001**	2.6 (1.5–4.6)	**0.001**
Other	19 (66)	27 (18)	73 (48)	1.2 (0.6–2.3)	0.69	1.1 (0.5–2.2)	0.82
Year culture specimen collected							
1999	52 (15)	17 (9)	83 (43)	Reference	**<0.0001‡**	Reference	**<0.0001**
2000	57 (16)	19 (11)	81 (46)	1.1 (0.4–3.0)		NS§	
2001	65 (19)	28 (18)	72 (47)	1.8 (0.7–4.5)		1.8 (0.9–3.7)	
2002	47 (13)	43 (20)	57 (27)	3.5 (1.4–8.9]		3.2 (1.5–6.9)	
2003	63 (18)	56 (35)	44 (28)	6.0 (2.4–14.3)		5.2 (2.5–10.5)	
2004	68 (19)	52 (35)	48(33)	5.1 (2.1–12)		4.4 (2.2–8.8)	

*MRSA, methicillin-resistant *Staphylococcus aureus*; HA, healthcare associated; CI, confidence interval; SD, standard deviation; SE, standard error; NS, not significant; **boldface** indicates significance.
†Multivariate analysis results reflect time in weeks.
‡Measured with the Cochran-Armitage test of trends.
§Was not a significant predictor in the multivariate model.

SCC*mec* IV element. The exception was an isolate that contained SCC*mec* IV that was resistant to gentamicin, clindamycin, and trimethoprim-sulfamethoxozole. The definition of the non-SCC*mec* IV phenotype was fulfilled by 10 (91%) of 11 isolates carrying the SCC*mec* II element. Phenotypic case definition of SCC*mec* type was highly correlated with the genotype confirmation of the SCC*mec* type phenotype (p<0.0001 by Fisher exact test).

Trend and Multivariate Analysis of the SCC*mec* type IV Phenotype

The proportion of MRSA isolates with the SCC*mec* type IV phenotype increased from 17% in 1999 to 56% in 2003 (p<0.0001, Figure). The proportion of isolates that were of the SCC*mec* type IV phenotype in 2004 (52%) was little changed from 2003 (Figure). In the multivariate model, independent predictors for having an SCC*mec* type IV phenotype isolate were wound source of culture (refer-

Table 2. Summary of genetic testing for 24 healthcare-associated MRSA blood isolates*†

No. isolates	SCC*mec*	*mec*I	*mecR1* (PB)	pvl	MLST	Clindamycin	Gentamicin	TMP-SMX	SCC*mec* type phenotype‡
8	IV	–	–	+	8	S	S	S	IV
1	IV	–	–	+	1	S	S	S	IV
2	IV	–	–	–	8	S	S	S	IV
1§	IV	–	-	–	8	R	R	R	II
5	II	+	+	–	5	R	R	S	II
4	II	+	+	–	5	R	S	S	II
1	II	+	+	–	8	R	S	S	II
1§	II	+	+	–	5	S	S	S	IV
1¶	-	–	–	–	5	R	S	S	II

*MRSA, methicillin-resistant *Staphylococcus aureus*; PB, penicillin-binding region; MLST, multilocus sequence typing; R, resistant; S, susceptible; TMP-SMX, trimethoprim-sulfamethoxazole.
†All isolates were associated with *mecA* and *ccr2* genes.
‡Phenotype: as defined by the study case definition, according to antimicrobial drug susceptibilities (see Methods).
§SCC*mec* phenotype did not match genotype.
¶SCC*mec* was not located for this isolate.

ent group was sputum source, OR 2.6, 95% CI 1.5–4.6, p = 0.001), culture obtained in less time after admission, (OR 0.88 per week, 95% CI 0.8–0.98, p = 0.02), and year of culture acquisition (p<0.0001) (Table 1).

Discussion

In many urban centers worldwide, infections due to MRSA account for a large proportion of CA–*S. aureus* infections; in some communities MRSA accounts for more than half of CA–*S. aureus* infections (*6*,*8*–*10*,*32*). There have been reports of strains frequently associated with community outbreaks causing HA infections, but they have been mostly limited to case reports or case series (*17*–*19*). To our knowledge, ours is the first investigation quantifying the rise of MRSA isolates typical of CA disease to become the predominant strain of HA-MRSA (i.e., accounting for >50% of MRSA strains) within the hospital setting. Remarkably, at our institution the number of HA-MRSA isolates that have a CA phenotype, which previously was uncommon, now is >50%.

Our analysis found 3 significant risk factors for an SCC*mec* type IV phenotype MRSA culture. First, patients with MRSA cultures from a wound source were more likely to have the SCC*mec* type IV phenotype. This finding may be understandable, given that the most common clinical syndrome described with CA-MRSA infections has been skin and soft tissue infections (*10*,*33*). In addition, 75% of CA-MRSA isolates that were genotyped carried the *pvl* gene, which has a strong association with skin and soft tissue infections (*33*). A second risk factor for the SCC*mec* type IV phenotype was a shorter length of hospital stay before MRSA culture. This association may be due to the increased severity of illness and coexisting conditions in patients with a longer hospital stay, factors that have been commonly associated with the traditional (non-SCC*mec* type IV) HA-MRSA infections. However, measures of severity of illness and coexisting conditions were not captured in this investigation. A third risk factor was a later year of culture collection; the likelihood of SCC*mec* type IV phenotype peaked in 2003. The rise of these isolates in our hospital may be from CA-MRSA isolates brought in from colonized persons from the community. CA-MRSA infections in Los Angeles County have rapidly become common and now exceed the frequency of those caused by CA–methicillin-susceptible *S. aureus* (*34*). Alternatively, the rise of SCC*mec* type IV isolates may be a result of spread throughout our hospital by the usual means of dissemination in a healthcare setting (e.g., hands of healthcare workers, contaminated environment) (*35*) or possibly by a combination of factors.

Exactly why the SCC*mec* type IV strains are successful in hospital settings such as ours and others (*20*) is unknown. Some evidence indicates that SCC*mec* type IV strains may be more "fit" than SCC*mec* types II/III that contain HA-MRSA isolates. Compared with methicillin-susceptible *S. aureus*, isolates containing SCC*mec* type II/III replicate more slowly in vitro (*36*). Okuma et al. found that CA-MRSA isolates that contain SCC*mec* type IV replicate more rapidly than these traditional HA-MRSA strains and argued that CA-MRSA may have enhanced ecologic fitness compared with SCC*mec* type II/III isolates, perhaps due simply to a shorter doubling time (*37*). Given the vulnerable population within the hospital setting, it is unclear how infections with isolates that contain SCC*mec* type IV will differ in symptoms and severity from those caused by traditional HA-MRSA isolates. On the basis of our study and other somewhat similar reports (*20*), concern is rising that USA300 strains may overtake the traditional HA-MRSA strains in many hospital and healthcare settings.

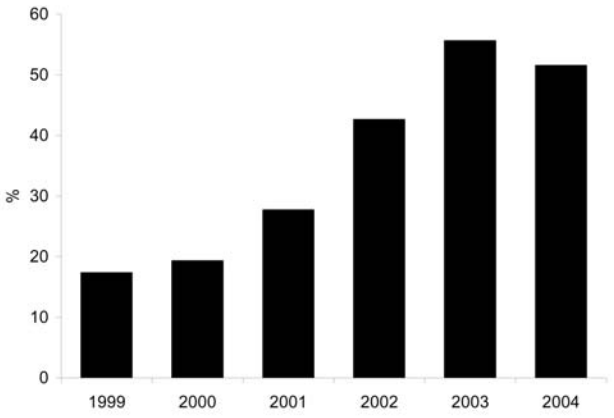

Figure. Percentage of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates among healthcare-associated MRSA isolates that are SCC*mec* type IV phenotype, 1999–2004.

Our investigation had some limitations. First, the analysis was retrospective and thus it was not possible to prospectively identify patients with HA infections and compare them with patients with CA infections. Although, by means of a chart review of a subset of patients who were selected by the criteria of a MRSA culture obtained ≥72 hrs after admission, none of these infections fulfilled the CDC definition of a CA-MRSA infection (*31*).

A second limitation was that our case definition was based on phenotypic criteria because nonbloodstream isolates had been discarded and the SCC*mec* type could not be validated. Traditionally, most HA-MRSA isolates in the United States carry SCC*mec* type II (and to a lesser extent, SCC*mec* type III) that encodes resistance to β-lactam antimicrobial agents bleomycin, macrolide-lincosamide-streptogamin B, aminoglycosides, and spectinomycin (*38*). Gentamicin resistance occurs in most strains that carry the SCC*mec* type II element but is conferred by the *aac6′–aph2″* gene elsewhere on the chromosome and is

frequently carried by transposon Tn*4001* (*11*,*16*). Therefore, to select for isolates that did not confer a phenotype typical of healthcare-associated or non-SCC*mec* type IV–containing isolates, the SCC*mec* type IV phenotype was defined as isolates that were resistant to oxacillin and susceptible to gentamicin, clindamycin, and trimethoprim-sulfamethoxazole.

Some banked isolates did not grow, and in 1 isolate we could not detect an SCC*mec* element. Of note, stored isolates may lose their SCC*mec* elements over time (*39*), which may explain our findings. Nevertheless, over the 6-year observation period of our investigation, among isolates, the phenotype and genotype definition of SCC*mec* type were in agreement for >90% of isolates. Thus, we were able to validate our case definition of an HA-MRSA isolate with SCC*mec* type IV phenotype using both chart review and SCC*mec* typing.

A third limitation of our investigation was that we were able to recover only bloodstream isolates, a subset of strains that are small and potentially nonrepresentattive. Whether the relationship of phenotype to genotype is similar for bloodstream and nonbloodstream infections is unclear. A fourth limitation is that all of the patients were from 1 institution and, therefore, may only reflect local trends. However, as previously mentioned, reports of isolates associated with the CA-MRSA infections causing HA infections are growing (*17*–*20*).

In summary, we found that over a 5-year span, MRSA with a CA-MRSA phenotype has become the most common cause of HA-MRSA infections in our institution. This finding has important implications for MRSA epidemiology, infection control practices, and empiric antimicrobial drug selection.

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Dr Maree is a clinical and research fellow in Infectious Diseases at UCLA Medical Center. She is currently studying community-associated MRSA infections and completing her PhD in epidemiology at the University of California, Los Angeles, School of Public Health.

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Address for correspondence: Loren G. Miller, Harbor-UCLA Medical Center, Division of Infectious Disease, 1000 W Carson St, Box 466, Torrance, CA 90509, USA: email: lgmiller@ucla.edu.

Subclinical Infection with Avian Influenza A (H5N1) Virus in Cats

Michael Leschnik,* Joachim Weikel,† Karin Möstl,* Sandra Revilla-Fernández,† Eveline Wodak,† Zoltan Bagó,† Elisabeth Vanek,† Viviane Benetka,* Michael Hess,* and Johann G. Thalhammer*

Avian influenza A virus subtype H5N1 was transmitted to domestic cats by close contact with infected birds. Virus-specific nucleic acids were detected in pharyngeal swabs from 3 of 40 randomly sampled cats from a group of 194 animals (day 8 after contact with an infected swan). All cats were transferred to a quarantine station and monitored for clinical signs, virus shedding, and antibody production until day 50. Despite unfamiliar handling, social distress, and the presence of other viral and nonviral pathogens that caused illness and poor health and compromised the immune systems, clinical signs of influenza did not develop in any of the cats. There was no evidence of horizontal transmission to other cats because antibodies against H5N1 virus developed in only 2 cats.

Avian influenza has attracted worldwide attention because highly pathogenic avian influenza virus subtype H5N1 can cause fatal infections in humans (*1*) and other mammals (*2*). Domestic cats and wild cats in a zoo have reportedly shown severe clinical signs and they may die of natural or experimental infections (*3–7*). Ingestion of infected birds was assumed to be the route of transmission in cats. However, horizontal transmission by experimentally infected cats has been demonstrated (*3*) and was also assumed under natural conditions in tigers in Thailand (*8*). No data are available on nonlethal outcomes of H5N1 infection in cats and whether horizontal transmission between feline hosts occurs under natural conditions. Also unknown is whether domestic cats play a role in the epidemiology of avian influenza, which could be an undefined hazard for poultry and humans (*9*).

*University of Veterinary Medicine, Vienna, Austria; and †Austrian Agency for Health and Food Safety Institute for Veterinary Disease Control, Mödling, Austria

During the first weeks of 2006, moribund or dead birds infected with avian influenza (H5N1) were found near water in Germany, Slovenia, and Austria. On February 14, 2006, a sick swan was found near the Mur River in Austria and transported to an animal shelter in Graz, Austria, where it died within 24 hours (day 1). PCR and egg culture identified avian influenza virus (H5N1) in the swan and in 13 of 38 other culled birds (swans, ducks, chickens) (day 4) housed with the swan at the same time. Only the swan developed clinical signs of disease. On day 4, the poultry area was disinfected after all 38 birds were removed.

In the same shelter were 194 cats; most had access to an outdoor enclosure near the poultry area and were separated from the birds by a wire-mesh fence. On several occasions, 1 or 2 unidentified cats were observed climbing the fence and entering the poultry area. Ingestion of birds by cats was not observed. Austrian authorities ordered random sampling of the cat population at the shelter because of spatial proximity of poultry and cats and the possible exposure of cats to infective debris of the birds. The bird area was left unoccupied while the cats were under observation. The purpose of this study was to monitor health status and possible transmission within a large cat population with proven natural exposure to H5N1 influenza virus.

Materials and Methods

Pharyngeal swabs of 40 cats were sampled (*10*) on day 8 and tested for H5N1 virus by PCR; positive results were obtained for 3 cats (cats 1, 2, and 3). All positive results were confirmed at the OIE reference laboratory in Weybridge, United Kingdom. All PCRs for H5N1 were conducted at the Agency for Health and Food Safety in Mödling, Austria. Daily physical examination by veterinarians showed no signs of influenza in any cat on days 4–21. In a follow-up examination on day 15, 0 of 34 cats

of the 40 cats previously tested (on day 8) were positive for H5N1 virus in pharyngeal swabs. In 3 cats that had died during this period, necropsy showed no evidence of infectious respiratory disease, and PCR results for influenza virus were negative.

On day 22 after the H5N1-infected swan was put in the animal shelter, 167 cats (5 kittens 4–6 months of age and 162 adults) were still available for further observations. Three cats had died and 24 other cats had been placed in private households. Before discharge from the shelter and within 1 week thereafter, all of these cats were examined and no abnormal health status was observed.

A total of 167 cats were transported in small groups in ≈50 containers for 12 h from the animal shelter to a quarantine area and housed in 2 separate groups from day 22 until day 50. Average floor space for each cat was ≈1.4m^2. The larger group contained 139 cats (including cats 1 and 2); the smaller group contained 28 cats. Cat 3 was not available for further examination because it was healthy before leaving the shelter and, to our knowledge, did not die. The smaller group was always separated from the larger group and was kept indoors at the animal shelter in Graz. In the quarantine area, the 167 cats were housed in 2 closed rooms, without any activity restriction, and had free access to food and water. Routine physical examination, including auscultation of the chest, was done on days 22, 29, and 50 for all cats at the quarantine station. In case of an obvious health problem, clinical signs were monitored by daily physical examination and serologic testing. The litter pans of the cats and floors of the quarantine areas were cleaned every day and disinfected every other day.

On days 22 and 29, pharyngeal and rectal swabs were obtained and transported in phosphate-buffered saline containing antimicrobial drugs (*10*). Swabs were obtained with special care to avoid any contact with the environment and were transferred immediately into tubes containing transport media. Blood was obtained on days 22, 29, 36, and 50. To facilitate physical examinations and collection of samples, we gave mild general anesthesia (propofol and midazolam) to all cats on day 29 (Table).

Pharyngeal and rectal swabs were examined for the matrix gene of influenza A virus by using a real-time reverse transcription–PCR (RT-PCR) according to the method of Spackman et al. (*11*). To screen for additional infections that might influence the health and immune status of the cats, we obtained 64 additional pharyngeal swabs on day 29 from cats with upper respiratory symptoms and tested them for nucleic acids specific for feline herpesvirus 1 (FHV-1)– and feline calicivirus (FCV). Real-time RT-PCR for FCV was conducted in a volume of 25 μL (22 μL reaction mixture and 3 μL template) in the Real-Time PCR system 7300 (Applied Biosystems, Foster City, CA, USA). The reaction mixture was prepared following the manufacturer's instructions of a commercially available kit (SuperScript III Platinum One-Step Quantitative RT-PCR Kit, Invitrogen, Carlsbad, CA, USA). This mixture contained 10 pmol/L of each primer (forward primer: 5′-AGTGGCATGACCGCCCT-3′, reverse primer: 5′-CGTTAGCGCAGGTTGAGCA-3′), and 5 pmol/L of probe (5′-FAM-CACTGTGATGTGTTCGAAGTTTG AGCA-TAMRA-3′). The cycler scheme consisted of 2 pre-PCR steps of 50°C for 15 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 s and 60°C for 30 s. Cycle threshold values were calculated by using PCR 7300 software (Applied Biosystems). FHV-1 nucleic acid was detected by PCR as described by Reubel et al. (*12*) and Stiles et al. (*13*).

Plasma samples were tested for feline leukemia virus (FeLV) antigen and antibodies against influenza virus A (H5N1), feline immunodeficiency virus (FIV), and feline coronavirus (FCoV). Antibodies to influenza virus were detected with a hemgglutination inhibition test according to the procedures of the World Organisation for Animal Health (*14*), FeLV antigen was detected by using an

Table. Time scale of sampling and course of influenza A virus H5N1 hemagglutinating antibody titer in cats 1, 2, and 4, Austria, 2006*

Day	Timeline events	No. pharyngeal swabs	No. rectal swabs	No. blood samples	H5N1 antibody titer
1	H5N1 virus–positive swan dies				
4	All poultry culled (38); 13 positive				
8	3 cats positive	40			
15	All cats negative	34			
22	Quarantine starts	160	160	14	Cat 1, 64 Cat 2, negative Cat 4, ND
29	Examination under anesthesia	All cats (164)	164	164	Cat 1, 128 Cat 2, negative Cat 4, 256
36				28	Cat 1, 128 Cat 2, negative Cat 4, ND
50	Cats 1 and 4 humanely killed for necropsy			All cats (155)	Cat 1, 256 Cat 4, 256

*ND, not done.

ELISA (ViraCHEK/FeLV, Synbiotics Corporation, San Diego, CA, USA), and antibodies to FIV were detected by using an immunomigration test (Witness FIV, Synbiotics Corporation). Three dilutions (1:10, 1:100, and 1:400) of each plasma sample were tested for antibodies to group 1 coronaviruses by a modified indirect immunofluorescence assay (*15*). Conjunctival, pharyngeal, and rectal swabs were cultured for pathologic bacterial infections (*16*).

Two cats that seroconverted for H5N1 virus (cats 1 and 4) were humanely killed on day 50. Necropsy was performed on these 2 cats and on 12 other cats that had died during the observation period; organ homogenates (lung, liver, brain, trachea, tonsils, stomach, spleen, and pancreas) were tested for influenza virus–specific nucleic acids for each cat.

Results

H5N1 virus–positive cats (1 and 2) and H5N1 virus antibody–positive cats (1 and 4) did not show any signs of influenza virus–associated illness after the swan had been placed in the animal shelter (days 1–50). Upper respiratory symptoms (laryngitis, bronchitis, and tracheitis) were evident in 30 cats, bronchopneumonia in 40 cats, diarrhea in 7 cats, mucosal lesions in 37 cats, and traumatic wounds and lesions in 10 cats. However, for each cat with clinical symptoms that might have been associated with influenza infection, another specific etiologic reason for illness could be documented. Pathomorphologic examination showed no lesions associated with respiratory infection in cats 1 and 4 or in any other cat that had died before day 50. Influenza A virus–specific nucleic acids were not detected in any organ sample tested by PCR. Likewise, all pharyngeal and rectal swabs obtained at the quarantine station were negative for influenza A virus by PCR. Antibodies against influenza virus A (H5N1) were detected in 2 cats (1 and 4, Table) with titers 256 on day 50 in both cats.

Cats 1, 2, and 4 had negative test results for FeLV and FIV, but all 3 cats had high antibody titers against FCoV. FCV was detected in the swab from cat 2, and a double infection with FCV and FHV-1 was detected in cat 4. Clinical, bacteriologic, and virologic tests identified infection with FeLV in 15 cats, FIV in 12 cats, and antibodies against FCoV in all but 1 cat. A total of 44 swabs showed positive results for FCV-specific nucleic acids, 4 for FHV-1; 13 samples showed a double infection with FCV and FHV-1. Some pathologic bacterial infections of the respiratory and digestive system were confirmed by swab cultures.

All veterinarians and staff members at the animal shelter and at the quarantine area were clinically monitored for any influenzalike symptoms. Because results of this monitoring were unremarkable and virus excretion by the cats was not detected, serologic tests were not conducted for these persons.

Discussion

This is the first description of an asymptomatic infection with highly pathogenic H5N1 influenza virus in domestic cats. Although infection was detected in a group of cats by positive PCR results for pharyngeal swabs in 3 cats and seroconversion in 2 cats, there was no evidence for influenza-associated disease. This finding contrasts with reports documenting cats with rapidly and developing and fatal disease caused by influenza A virus subtype H5N1 (*3*–*5*,*7*). High fever, depression, severe pneumonia, pulmonary edema, nonsuppurative encephalitis, and sudden death were observed after natural (*5*) or experimental infections (*3*,*7*). Infection with influenza virus H5N1 was shown to cause severe lower respiratory tract disease as well as systemic disease that affected many organs outside the respiratory tract, which could explain the increased pathogenicity of this virus for other organ systems (*3*,*17*).

During the observation period, episodes of sickness including respiratory symptoms (mild dyspnea, conjunctival, and nasal discharge), oral mucosal lesions, and diarrhea were observed in cats in both groups in the animal shelter and in the quarantine station. A long (12 hours) and uncomfortable transport to the quarantine area, social distress caused by high population density, repeated restraint for examinations and sample collection, and multiple infectious agents may have caused such a high level of illness. Twelve cats died or were humanely killed while in a moribund state between days 22 and 50. All showed signs of disease other than infection with influenza virus A and died of feline infectious peritonitis, cardiomyopathy, enteritis, or nephropathy; none tested positive for H5N1 virus.

During the observation period from days 22 to 50, excretion of virus was not detected in the pharynx or feces. Positive results were observed only on day 8 for 3 of the randomly sampled swabs. Therefore, viral shedding is assumed to have lasted <2 weeks in cats 1 and 2. In 1 study, no information was reported on the duration of virus shedding because only severe illness with a lethal outcome was reported or the cats were killed 7 days after experimental infection (*3*). Because seroconversion was confirmed in only 2 animals, horizontal transmission within the group of 194 cats is unlikely. This conclusion is consistent with the finding that no virus shedding could be demonstrated after day 8, but it contrasts with the results of Rimmelzwaan et al. (*3*), who demonstrated horizontal transmission from experimentally infected cats to sentinel cats, and results of studies in mice and ferrets (*18*,*19*). After infection by the oral or intratracheal route, cats developed viremia; virus spread into different tissues and was excreted in feces and saliva (*7*). High viral load and differences in virus strains could result in different host reactions.

The reason for limited horizontal transmission in our study could be low-level virus shedding by the initially infected cats. Initial virus load, route of virus uptake, and the immune system of the cat may affect infection and disease. Otherwise, the lack of illness would be unusual because several cats in the study had immunodeficiencies caused by other infectious diseases (*20*,*21*).

An asymptomatic infection confirmed by seroconversion is assumed for cats 1 and 4. The situation for cat 2 is not as clear. It is unlikely that the positive PCR result in the swab sample from cat 2 is due to contamination and is a false-positive result. Conversely, infection could not be confirmed by seroconversion. It remains unclear whether ongoing infection could be stopped (possibly by interferons) or whether the cat did not produce sufficient amount of antibodies. Little information is available on immune responses after infection with influenza virus H5N1 in cats.

H5N1 virus can cross species barriers (*22*) and infect new hosts. Transmission from poultry to mammals, between cats (*3*,*5*,*7*,*8*), and between humans (*23*) indicates 2 routes of virus uptake under natural conditions. The first is orally by ingestion of raw poultry, and the second is transmission by contact with feces or saliva of infected animals. In our study, virus transmission from infected poultry to cats must have occurred from days 1 to 4. Uptake of H5N1 virus by ingestion of infected poultry can be ruled out. We observed only some cats entering the area where the birds were housed. Therefore, the most likely route of transmission for these cats is contagious fecal contamination of the hair and oral uptake during grooming. However, we cannot exclude aerosolization of the virus as a route of transmission.

Until recently, the avian flu situations in Asia and Europe appeared to differ. In Asia, large numbers of poultry have been infected and culled. Human and feline cases are mainly associated with close contact with infected poultry or ingestion of contaminated meat that was not sufficiently cooked. In Europe, mainly wild aquatic birds were infected, and only a few turkey farms were affected by H5N1 infection. Because direct contact with poultry is more limited in Europe than in Asian countries and the main source of food for cats in Europe is either commercial cat food or wild rodents and small birds, virus uptake during hunting and ingestion of poultry and aquatic birds is unlikely. Large aquatic birds are normally not a major source of food for cats, although infected birds may have caused the deaths of 3 cats found on the island of Ruegen, Germany (*4*).

We have shown that under natural conditions infection of cats with influenza virus H5N1 may occur after contact with infected birds or their excrement without inducing clinical disease. However, horizontal transmission between cats was not observed, although infected cats had been introduced into a large cat population that had other viral and bacterial infections and lived under stressful conditions. Avian flu infection in cats is rarely documented and there is no evidence to date that cats are responsible for transmitting the virus to humans. Although this study does not rule out H5N1 infection leading to disease and possible transmission to other mammals and birds by domestic cats under natural conditions, without ingestion of infected birds, cats do not represent a major risk in the epidemiology of H5N1 influenza. The risk posed by cats could change because the virus can rapidly undergo genetic mutation and reassortment, and efforts should be made to minimize contact of domestic cats with infected birds. To have better insights into whether cats represent a potential risk in the epidemiology of H5N1 influenza, more detailed knowledge is needed about the role of viral load, virus uptake, and immune mechanisms of the host on the outcome of infection with H5N1 influenza virus.

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Dr Leschnik is a veterinary staff member of the Clinic for Internal Medicine and Infectious Diseases, University of Veterinary Medicine, Vienna, Austria. His research interests include small animal neurology and infectious diseases.

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Address for correspondence: Michael Leschnik, Clinic of Internal Medicine and Infectious Diseases, Clinical Department of Small Animals and Horses, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria; email: michael.leschnik@vu-wien.ac.at

Human African Trypanosomiasis in a Rural Community, Democratic Republic of Congo

Pascal Lutumba,*† Eric Makieya,‡ Alexandra Shaw,§ Filip Meheus,¶ and Marleen Boelaert†

According to the World Health Organization, human African trypanosomiasis (HAT) (sleeping sickness) caused the loss of ≈1.5 million disability-adjusted life years (DALYs) in 2002. We describe the effect of HAT during 2000–2002 in Buma, a rural community near Kinshasa in the Democratic Republic of Congo. We used retrospective questionnaire surveys to estimate HAT-related household costs and DALYs. The HAT outbreak in Buma involved 57 patients and affected 47 (21%) households. The cost to each household was equivalent to 5 months' income for that household. The total number of HAT-related DALYs was 2,145, and interventions to control HAT averted 1,408 DALYs. The cost per DALY averted was US $17. Because HAT has a serious economic effect on households and control interventions are cost-effective, considering only global burden of disease rankings for resource allocation could lead to misguided priority setting if applied without caution in HAT-affected countries.

Human African trypanosomiasis (HAT), or sleeping sickness, is a vectorborne disease caused by the parasite *Trypanosoma brucei*. East African HAT, an acute syndrome, is caused by *T. b. rhodesiense*; West African HAT, a disease with a more protracted course, by *T. b. gambiense*. HAT is a major public health problem in sub-Saharan Africa, where it affects mainly the rural poor; the most recent prevalence estimates from the World Health Organization (WHO) are 50,000–70,000 cases, based on a total number of 17,500 new cases reported per year worldwide (*1*). Odiit et al. calculated that 39% of HAT cases and 92% of deaths caused by HAT were unreported in a *T. b. rhodesiense* –endemic area (*2*). In the absence of appropriate treatment, HAT infection inevitably leads to death (*2*). Although historic accounts of devastating epidemics exist (*3*), the real effect of HAT on communities has not been well documented.

WHO estimates that current HAT control activities reach only 10% of persons at risk. HAT control requires considerable resources, and budgets depend mainly on international donors (*4*). Resource allocation by the latter is often guided by criteria such as burden of disease expressed in disability-adjusted life years (DALYs) as proposed by Murray (*5*,*6*). This measure is the sum of years lost due to premature death and years lost due to disability. According to WHO global burden of disease estimates, HAT caused 1.5 million DALYs in 2002 (*7*), which ranks it much lower than most infectious diseases in Africa but high among parasitic diseases. The use of DALYs for priority setting has provoked a lot of discussion, and caution is needed when using them as a tool for planning and resource allocation (*8*). The current estimate of HAT DALYs is global and does not take into account local and regional aspects. HAT has a clustered distribution, and at times, local attack rates exceed 10%, but HAT is treated in the same way as diseases that have relatively homogeneous attack rates (*9*). Moreover, differences in the course of the disease caused by *T. b. rhodesiense* in East Africa and that caused by *T. b. gambiense* in West and Central Africa are ignored.

Another way to express the effect of the disease on communities is to examine its economic effect at the household level (*10*). The advantage of this approach is that it can enhance our understanding of how disease would cause further impoverishment of the household and even hamper

*Programme National de Lutte contre la Trypanosomiase Humaine Africaine, Kinshasa, Democratic Republic of Congo; †Institute of Tropical Medicine, Antwerp, Belgium; ‡University of Kinshasa, Kinshasa, Democratic Republic of Congo; §AP Consultants, Andover, United Kingdom; and ¶Royal Tropical Institute, Amsterdam, the Netherlands

control efforts. Only a few studies concerning the economic effects of HAT have been undertaken. Gouteux et al. measured the days of productivity lost in Niari (Brazzaville, Republic of Congo) and estimated the household cost to be 58,000 CFA francs (≈US $100) (*11*). These authors suggested that this cost may have contributed to patients' frequently refusing to seek treatment, although treatment is provided for free by the health services. Despite the fact that almost all HAT control programs subsidize the cost of drugs and hospitalization, often patients either do not seek treatment or only do so a long time after their diagnosis or when their symptoms become more acute (*12*). Robays et al. showed how the enforced rest period of 6 months after treatment leads asymptomatic patients to refuse treatment for fear of substantial loss of income (*13*).

The indirect cost (i.e., all HAT-related costs incurred by the household that are not for diagnosis, drugs, or care) is a real obstacle that prevents persons from seeking treatment (*11*). At a time when the world considers the fight against poverty a top priority, we think that the practice of setting priorities for healthcare based on aggregate figures at the global level should be reexamined. We put forward the hypothesis that neglected diseases such as HAT compromise the economic development and well-being of populations in HAT-endemic regions to a much higher degree than we are led to believe by lists of DALYs established globally (*14*). The aim of our study was to document the effect of HAT caused by *T. b. gambiense* during 2000–2002 in a rural community in the Democratic Republic of Congo (DRC) that was affected by a single outbreak.

Methods

Study Area

The study was conducted in the HAT focus of Buma, in the N'sele health district in DRC, 35 km south of Kinshasa. Buma consists of several villages, including Buma-centre (population 1,000) and Kimpolo (population 300). The socioeconomic situation in each village is similar. Agriculture is the main economic activity and produces maize, charcoal, firewood, vegetables, and leaves for packaging of manioc. These products are sold in the markets of urban Kinshasa.

The district medical officer of N'sele health district declared the first suspected cases of HAT near Buma in 2000. The national program soon corroborated that health facilities had been detecting HAT cases through passive case finding and had been reporting cases from that area for some time. In 2001, the control program sent a mobile team to conduct an active case-finding campaign that was repeated the following years. Screening in 2001 and 2002 confirmed 77 HAT cases in the Buma foci: 20 in Buma-centre and 57 in Kimpolo.

Estimating Cost of Illness

All households of Buma and Kimpolo in which ≥1 HAT case was confirmed by the mobile teams or the permanent health facilities from January 1, 2001, through December 31, 2002, were eligible. HAT was confirmed by direct microscopic examination of lymph node aspirate, fresh blood film, or thick blood film. In February 2003, we identified all households with confirmed cases of HAT by using a list provided by the village head, information provided by the inhabitants of Buma and Kimpolo, and data from the epidemiologic surveillance of the Programme National de Lutte contre la Trypanosomiase Humaine Africaine (PNLTHA) in DRC. Households were visited to ask members to participate in the study. The survey was conducted after working hours and during the weekend by 3 enumerators trained by the principal investigator. A pretested questionnaire was used for interviewing the patients, their caregivers, or any other member of the household who could provide useful information. All case-patients in a household were interviewed. We collected data on residence, age, sex, stage of the disease, number of working days lost by the patient and caregiver, and expenses incurred because of HAT. For households with ≥1 case-patient, the time of only 1 caregiver was taken into account. Data on costs were collected in Congolese francs (FC) and converted into US$ at the market rate for 2002, which was 330 FC for 1 US$. The economic cost of HAT comprises household costs and costs to the health system minus transfers from the households to the health system, to avoid double-counting. In this study, household costs included consultation fees, cost of travel, laboratory costs including all household expenses for diagnostic tests, and the cost of hospitalization (all expenses for hospitalization as well as food for the patient and caregiver). Treatment costs included cost of drugs, injections, and small material such as syringes and needles. The total cost of HAT for the household was estimated as the sum of all costs mentioned above and the value of all the days of work lost after HAT confirmation. The value of each day of work lost was estimated separately for each person, according to monthly production of the household.

Estimating Household Monthly Income

Estimation of monthly household income was based on agricultural production data. To validate this information, these data were compared with household expenses and financial aid received. Children's production was considered to be zero even if the children helped with household tasks.

Because the main activity is agriculture and the work in the fields is done by the whole family, quantifying the contribution of the sick person to the household production is difficult. In our calculations, we considered that the

whole household was affected, on the assumption that the entire family's activities are disturbed if 1 member is affected by HAT. The income losses are calculated for individual caregivers and patients and then examined as a percentage of total household income.

Estimating DALYs

In August 2003, we organized a retrospective survey of illness and death among all households of Buma-centre and Kimpolo. Our objective for this second, exhaustive household survey was to measure HAT-related DALYs for confirmed HAT case-patients interviewed previously and for other possibly nondetected HAT-related deaths in the community that were missed by the control program. A questionnaire was developed and pretested in Kimwenza, another rural community near Kinshasa. We collected information for a 3-year recall period, between 2000 and 2002. To help participants determine the recall period, we constructed a local calendar with a number of key events, including the attack of Tutsi rebels on the city in August 1998 and the death of president Laurent Désiré Kabila in January 2001.

A team of 3 physicians visited all households of Buma and Kimpolo to invite them to participate in the survey. The head of the household or the person in charge was interviewed. Information concerning residence, composition of the household, and economic activities was collected for each household. For each household member we collected information about age, sex, and disease episodes experienced during the recall period. The same information was collected for household members who had died between 2000 and 2002; verbal autopsies (caregiver interviews) were used to help determine the cause of death (*15*). The interviewers used the following definition for a HAT-related death: a person who died after a protracted disease with loss of weight but without cough or diarrhea; with repeated bouts of fever; and with or without neuropsychiatric symptoms such as somnolence, psychosis, and other behavioral problems. The patient record, if available, was examined to verify findings.

For each HAT case and HAT-related death, we documented the degree of disability caused by the disease before, during, and after treatment. The degree of disability was based on the scale proposed by Murray (*5*), for which we adapted the list of activities for the Congolese setting. To corroborate our interview results, we checked other sources of information for illness and death in this community, consulted administrative documents of the neighborhood office, discussed with community leaders, and visited 2 graveyards in Buma and Kimpolo to obtain an exhaustive list of deaths and HAT cases.

Calculating DALYs

Calculations were based on the recommendations of Murray (*5*). The total number of DALYs caused by a specific disease is defined as the sum of years lost by the premature death (years of life lost [YLL]) of patients and the number of years lived with the disability (YLD) adjusted for the severity of the disability. We used the calculation method, discount rate of 0.03, age weighting factor of 0.04, and age weight modulating factor of 1 proposed by Fox-Rushby and Hanson (*16*).

YLD for each patient was estimated from the questionnaire, and the results were combined to obtain the total number of YLD. To estimate YLL, age categories spanning 5 years were used.

The life tables for each age group were estimated by using Population Analysis Spreadsheets software (International Programs Center, Bureau of the Census, Washington, DC, USA). Data on age distribution, sex ratio, and crude mortality rate were derived from the 2004 international database of the US Census Bureau (www.census.gov/ipc/www/pas.html).

When these calculations are used, the number of DALYs may be underestimated because of the poor sensitivity of the active case-finding rounds (*12*,*17*). Our exhaustive household survey and interviews with health workers did not entirely correct for this bias because the case definition used for verbal autopsy was specific and mainly applied to patients with more advanced disease. We tried to correct for this by supposing that each undetected case-patient will eventually die. On the basis of observations of Robays et al., we estimated that the effectiveness of the active case finding was 60% per round and that 40% of the cases could not be detected (*12*).

Quantifying DALYs

We did an exhaustive census of DALYs caused by HAT in 2 villages affected by the 2000–2002 outbreak. We first used our observations to calculate DALYs caused by HAT in this community. Then we estimated DALYs caused by HAT in absence of any intervention in the same community. To do this we needed to accept a number of assumptions. On the basis of the work of Fèvre et al., we estimated that without treatment the median survival time for a person infected by *T. b. gambiense* is 36 months when in the first stage of the HAT disease and 12 months when in the second stage (*18*). The average degree of disability of persons in the first or second stages of HAT was calculated by using the weightings developed by Murray et al. (*5*) adapted to the DRC context. We compared the existing intervention measures (active case finding followed by treatment) to hypothetical nonintervention.

Data Analysis

We entered our data in an Access database (Microsoft Corp., Redmond, WA, USA). Data were analyzed with Excel (Microsoft Corporation) and EpiInfo 2002 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Results

We found 47 households (21% of all households) with ≥1 new HAT case diagnosed since 2000. We located 57 of the 77 HAT patients reported by PNLTHA in Bumacentre and Kimpolo (74%) during 2000–2002. Four persons died of HAT during this time in this community of 1,300 persons. Table 1 describes the household characteristics. All but 1 patient eventually sought treatment after varying time periods since diagnosis. Patient median age was 26 years (range 4–72 years), and 57% of patients were female. Fifty (87%) of the 57 cases were detected by the mobile team during active case finding. At the time of diagnosis, 36 (63%) were in the first stage of the disease. The median time of patient hospitalization was 10 days (range 7–45 days), and time after hospitalization (including enforced rest) was 90 days (range 30–270 days); time spent by caregiver during and after patient's hospitalization was 10 days (range 0–94 days). The percentages of out-of-pocket expenditures incurred by the 47 households in Buma for 57 HAT cases were as follows: indirect costs 94.55%, hospitalization 4.16%, treatment 1.11%, consultation 0.10%, and laboratory 0.09%. The median value of a day's work per household was US $1.2. The median cost of HAT case per household was US $163.98 (range US $32.30–$3,731.70). This cost represents 43% of the annual revenue of a household (an estimated US $384 [range US $0–$1,980]) and is based on agricultural production and small trade.

An attempt to identify HAT cases from before 2000 by using verbal autopsy and other methods was not successful, most likely because this outbreak was recent. The detailed evaluation of the total YLL caused by HAT is shown in Tables 2 and 3. With and without intervention, YLDs weighted for age would be 16 and 40, respectively, and YLLs weighted for age would be 721 and 2,104, respectively. A total of 2,145 DALYs (27 per case) would have occurred in this community had no intervention taken place. Under the current control strategy of repeated active population screening and treatment, the disease still caused 737 DALYs. We conclude that the intervention enabled 1,408 DALYs to be averted at a savings of US $17 per DALY. At a cost of US $301 for HAT control per case detected and patient cured, the total intervention for 79.8 cases (57 cases detected multiplied by a factor of 1.4, assuming 40% of cases remain undetected) was US $24,019.80.

Table 1. Characteristics of 47 households with human African trypanosomiasis patients, Buma, Democratic Republic of Congo, 2003

Characteristic	Value
Household size, no. persons	
Minimum	1
Median	5
Maximum	12
Interquartile range	3
Age of head of household, y	
Minimum	23
Median	45
Maximum	63
Interquartile range	12
Proportion of households with male head of household	87.2%
Proportion of farmers among heads of household	80.9%
Education level of heads of household	
None	8.5%
Primary	40.4%
Secondary	46.8%
University	4.3%

Discussion

Our study shows that HAT costs households in Buma the equivalent of 5 months of household income, despite the fact that HAT control activities are heavily subsidized. The cost for a patient with complications increases considerably, to as much as 17 months of household income. HAT complications concern mainly the central nervous system; patients with this complication face a substantial loss in productivity and, hence, revenue. The study shows that a large number of working days were lost after treatment for HAT, as the national program recommends a rest period of 6 months. This recommended rest period is not always adhered to exactly; some patients resume their activities after 30 days, but others scrupulously rest for the full period. This compulsory rest period contributes to the fear of a HAT diagnosis.

Our survey involved a limited number of patients in a rural district near the capital. Household incomes in more isolated districts are probably lower than those in Buma, and the effect of HAT on households is thus probably greater. Another limitation of the study was that household income was estimated on the basis of agricultural production. This estimation was validated by the estimation based on households' real expenses. Seasonal variation of agricultural production could affect our results. To better estimate loss of production, a prospective study comparing households with and without HAT would be necessary because the disease is chronic and weakens the household progressively.

Our figures are comparable to those of Gouteux et al., who calculated the average household cost of a HAT

Table 2. Estimation of HAT YLLs with intervention, Buma, Democratic Republic of Congo, 2004*†

Age at onset, y	No. cases detected	No. deaths detected	Undetected cases/deaths (at 40% underdetection)	Total deaths	Life expectancy, y	YLL age-weighted per death	YLL age-weighted	
							YLL‡	YLL§
0–5	1		0.4	0.4	55.2	34.4	0	13.7
6–10	4	1	1.6	2.6	55.7	36.0	36.0	93.6
11–15	9	1	3.6	4.6	52.6	35.4	35.4	162.8
16–20	8		3.2	3.2	48.5	33.5	0	107.3
21–25	4	1	1.6	2.6	44.5	31.0	31.0	80.6
26–30	9		3.6	3.6	40.8	28.1	0	101.2
31–35	3		1.2	1.2	37.0	25.0	0	30.0
36–40	5		2.0	2.0	33.2	21.9	0	43.7
41–45	2		0.8	0.8	29.5	18.8	0	15.0
46–50	7		2.8	2.8	25.8	15.9	0	44.4
51–55	2		0.8	0.8	22.1	13.1	0	10.5
56–60	1		0.4	0.4	18.5	10.5	0	4.2
61–65	1	1	0.4	1.4	15.1	8.2	8.2	11.5
66–70	1		0.4	0.4	12.0	6.1	0	2.5
Total	57	4	22.8	26.8			110.6	720.9

*HAT, human African trypanosomiasis; YLL, years of life lost; YLD, years lived with disability; DALYs, disability-adjusted life years.
†Discount rate = 0.03; age weighting factor = 0.04; age weight modulating factor =1; DALYs with intervention = 16 (YLD) + 721 (YLL) = 737; DALYs without intervention = 40 (YLD) + 2,104 (YLL) = 2,144.
‡Excluding undetected cases.
§Including undetected cases.

episode in 1987 in Niari to be US $100 (*11*). Difficulty finding funds to meet health expenses for HAT has been reported by Odiit et al. (*19*). Household cost studies have shown that rural populations are often incapable of finding the funds to make even small, symbolic payments for healthcare and disease prevention. In Kenya, households were not able to pay for an impregnated bed net, even at a reduced price (*20*). Poor households in Malawi required 32% of their income to cover expenses linked to malaria (*21*). In Tanzania, the household cost for tuberculosis (in addition to the cost of treatment [US $20]) varied between US $187 and US $1,457 (*22*).

Ours is 1 of few studies to analyze the economic effect of HAT at the household level. This aspect is rarely captured by public health analyses, which often remain at the level of quantifying illness and death. The socioeconomic effect of a severe disease such as HAT goes beyond these figures. During our study in Kimpolo, farmers told us how in the year 2000 they slaughtered all their pigs after the first cases of HAT were identified because a community health worker had advised them to do so to decrease the density of tsetse flies.

This study estimated that HAT would have caused 2,145 DALYs in the absence of intervention. The interven-

Table 3. Estimation of HAT YLL without intervention, Buma, Democratic Republic of Congo, 2004*†

Age at onset, y	Detected cases	Detected deaths	Undetected cases/death (at 40% underdetection)	Total deaths	Life expectancy, y	YLL age-weighted per death	YLL age-weighted	
							YLL‡	YLL§
0–5	1	0	0.4	0.4	55.2	34.4	0	13.7
6–10	4	3	1.6	4.6	55.7	36.0	108.0	165.6
11–15	9	9	3.6	12.6	52.6	35.4	318.5	445.9
16–20	8	8	3.2	11.2	48.5	33.5	268.3	375.6
21–25	4	6	1.6	7.6	44.5	31.0	186.0	235.6
26–30	9	6	3.6	9.6	40.8	28.1	168.6	269.7
31–35	3	6	1.2	7.2	37.0	25.0	150.0	180.1
36–40	5	3	2.0	5.0	33.2	21.9	65.6	109.4
41–45	2	4	0.8	4.8	29.5	18.8	75.2	90.2
46–50	7	4	2.8	6.8	25.8	15.9	63.4	107.8
51–55	2	5	0.8	5.8	22.1	13.1	65.4	75.8
56–60	1	1	0.4	1.4	18.5	10.5	10.5	14.7
61–65	1	1	0.4	1.4	15.1	8.2	8.2	11.5
66–70	1	1	0.4	1.4	12.0	6.1	6.1	8.6
Total	57	57	22.8	79.8			1,493.8	2,104.2

*HAT, human African trypanosomiasis; YLL, years of life lost.
†Discount rate = 0.03; age weighting factor = 0.04; age weight modulating factor = 1.
‡Excluding undetected cases.
§Including undetected cases.

tion carried out by the control program averted 1,408 DALYs at a cost of US $17 per DALY averted. These figures were based on a number of assumptions. When quantifying DALYs, we assumed that a patient would die after a median of 3 years (*18*). We also assumed, according to the work of Robays et al., that 40% of the real HAT cases remain undetected by 1 screening round and that these case-patients would inevitably die (*12*). However, in practice, these persons could be detected subsequently at fixed health facilities or during a second visit by a mobile team. The cost of the intervention per DALY averted falls within the ranges modeled by Shaw and Cattand (*23*). The US $17 cost per DALY averted is lower than for many health interventions (e.g., the cost per DALY of US $19–$85 for insecticide-treated bed nets for malaria control in sub-Saharan Africa) and places HAT control in the range of cost-effective interventions (*24*,*25*).

Nevertheless, the cost of treatment borne by households is considerable and can compromise the timely receipt of treatment. Household members take time to prepare themselves and mobilize resources, relying on the solidarity of the extended family, before they seek treatment for HAT. The high household cost may partly explain the low participation rate at the active screening session organized by the mobile teams (*12*).

We conclude that not only does HAT affect the health of the persons touched by the disease, but also it places a substantial hardship on the affected households. This effect can be fully evaluated only when taking into account specific local situations. Using a global DALYs ranking to set healthcare priorities may not capture the full effect of certain diseases in communities.

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Dr Lutumba is head of the research unit of the national sleeping sickness control program of DRC. He has extensive experience in sleeping sickness control in DRC and participates in clinical research programs on diagnosis and treatment of HAT.

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Address for correspondence: Marleen Boelaert, Epidemiology and Disease Control Unit, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium; email: mboelaert@itg.be

Methicillin-resistant *Staphylococcus aureus* ST398 in Humans and Animals, Central Europe

Wolfgang Witte,* Birgit Strommenger,* Christian Stanek,† and Christiane Cuny†

Methicillin-resistant *Staphylococcus aureus* of clonal lineage ST398 that exhibits related spa types and contains SCC*mec* elements of types IVa or V has been isolated from colonized and infected humans and companion animals (e.g., dog, pig, horse) in Germany and Austria. Of particular concern is the association of these cases with cases of nosocomial ventilator-associated pneumonia.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an infection control problem in hospitals worldwide, mainly associated with intrahospital and interhospital dissemination of particular epidemic clonal lineages of the *S. aureus* population (hMRSA; [*1*]). MRSA primarily associated with healthcare facilities may also be disseminated to the community through colonized medical staff or discharged patients. The emergence and spread of MRSA in the community during the past 5 years, independent of the healthcare setting and in the absence of typical risk factors for nosocomial MRSA infections, are matters of further concern. These community-acquired MRSA infections are less broadly resistant to antimicrobial agents than are healthcare-associated MRSA and often contain the determinants *lukS-lukF*, which code for Panton-Valentine leukocidin (*2*).

Even though MRSA has been known as a nosocomial pathogen for >30 years, its development in companion animals and livestock has been rare (*3*). Recent reports, however, have documented MRSA infections in animals such as horses from Canada (*4*) and Europe (*3*) and pets (*5*,*6*). Of particular interest is whether MRSA may be transmitted between animals and humans. MRSA of clonal lineage sequence type (ST) 22 is widely disseminated in human hospitals in the United Kingdom and Central Europe. The demonstration of this lineage among MRSA isolates from staff and from pets in a small animal referral hospital in United Kingdom suggests transmission between humans and animals (*5*). Nasal colonization of veterinary staff with MRSA (ST8) from infections in horses in a veterinary hospital was frequently observed in Canada (*4*), and it was also recorded in an Austrian university veterinary hospital where horses were affected by MRSA of clonal lineage ST254 (*3*).

We report on molecular characterization of MRSA, from sporadic infections in humans and in various animal species, that belong to clonal lineage ST398 according to multilocus sequence typing (MLST). These isolates were further characterized by *spa*-sequence typing (repeat polymorphism of the X-region of the *spa* gene) and by PCR for grouping of staphylococcal cassette chromosome *mec* (SCC*mec*) elements, which contain the *mecA* gene and of which at least 5 basic types have been described.

Methods

MRSA isolates from infections in humans and in animals were sent to the National Reference Center for Staphylococci at the Robert Koch Institute, Wernigerode Branch, in Germany, for typing by means of *Sma*I-macrorestriction pattern as well as *spa* typing. Selected isolates also underwent MLST. Four human isolates were grown from nasal swabs taken from the staff of a veterinary practice at Veterinary Analytical Center, Geesthacht, Germany. All isolates were primarily grown on sheep blood agar and confirmed by standard procedures as

*Robert Koch Institute, Wernigerode, Germany; and †Veterinary University, Vienna, Austria

S. aureus. Eleven additional MRSA specimens of lineage ST398 (1 isolate per patient affected) were found among 4,370 MRSA isolates from patients with recognized infections. These isolates were identified by indigestibility of their whole cellular DNA when subjected to *Sma*I-macrorestriction analysis. Animal isolates were collected from 1 dog and 1 foal at the Veterinary Analytical Center, Geesthacht, Germany; from 1 pig at the diagnostic laboratory of the Institute for Microbiology and Infectious Diseases, School of Veterinary Medicine, Hannover, Germany; and from 2 horses at the Department of Orthopaedics, Veterinary University, Vienna, Austria.

Procedures and primers for DNA extraction and PCR detection of resistance genes were as described previously (*6*). Macrorestriction patterns were determined by using lysis of cells, deproteinization and digestion of DNA (here by *Sma*I and *Apa*I), and pulsed-field gel electrophoresis (*7*).

The polymorphic X-region of the protein A gene (*spa*) was amplified and sequenced according to the Ridom StaphType standard protocol (www.ridom.org). The resulting *spa*-types were assigned by using the Ridom StaphType software package (Ridom GmbH, Würzburg, Germany). The BURP algorithm, implemented in the most recent Ridom StaphType software version, was used for cluster analysis of *spa* types (*7*).

Primers used for MLST correspond to the protocol as described previously (*8*), with the exception of the forward primer for *tpi*; we used the sequence tpif 5′-GCAT TAGCAGATTTAGGCGT-3′. Antimicrobial susceptibility testing was performed by broth microdilution, performed according to DIN 58940, Deutsches Institut für Normung (*9*). SCC*mec* elements of types I to IV were characterized by using a PCR approach, including a combination of different PCRs (*6*). To demonstrate SCC*mec*-elements of type V, we used primers type VF/type VR, as described by Zhang et al. (*10*), as well primer pair *ccr*C9f 5′-CACT-TAATCCATGTACACAG-3′ and *ccr*C-R (*10*).

The following set of primers was used for PCR for virulence-associated genes: *tst*, *sea, seb, sec, sed, see*, as described by Johnson et al. (*11*); for *lukS-lukF*, forward 5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′, reverse 5′-GCATCAAGTGTATTGGATAGCAAAAGC-3′; for *cna*, forward 5′-CGGTTCCCCCATAAAAGT GAAG-3′, reverse 5′-CCCATAGCCTTGTGGATTTG-3′. Annealing temperature was 55° C; cyclic scheme and further conditions were as reported previously (*6*).

Specimen collection, characterization of the isolates, data processing, and exchange of data were performed within the framework of German public health activities for infection control and prevention of MRSA dissemination. Ethical approval was obtained within this framework as well.

Results

Characteristics of the 20 MRSA isolates investigated are shown in the Table. All isolates share MLST ST398 with the allelic profile 3-35-19-2-20-26-39. Three different *spa*-types are obviously related (Figure). Types t11 and t34 may have been derived from each other by either deletion or duplication of 2 repeats; t1197 and t11 differ by a single nucleotide polymorphism. BURP analysis of these *spa*-types groups them as a separate cluster unrelated to other BURP clusters (*7*). A peculiarity of *S. aureus* of clonal lineage ST398 is the indigestibility of whole cellular DNA by restriction enzyme *Sma*I. Therefore, *Sma*I macrorestriction patterns generate only 1 large fragment because of protection by a novel DNA methylation enzyme (*12*). We also found poor digestion by the isoschizomeric enzyme *Xma*I. However, digestion by enzyme *Apa*I generated similar fragment patterns that differed at most by 3 fragments independent of *spa* types.

The 2 horse isolates from the Vienna veterinary university contained SCC*mec* elements of group IVa. For all other isolates investigated, PCR indicated SCC*mec*V. These findings suggest that MRSA of ST398 from horses are unrelated to the other isolates and probably have evolved independently by acquisition of a different SCC*mec* element.

In addition to *mecA*, all investigated isolates contained *tet*M; isolates from animals and humans from Lower Saxony also contained *ermA*. The nosocomial human and horse isolates contained *ermC*; in the horse isolates, aph2″-aac6′–mediating aminoglycoside resistance was demonstrated. PCR was negative for virulence-associated genes and for *lukS-lukF* (coding for Panton-Valentine leukocidin), *tst*, *sea*, *seb*, *sec*, and *sed*, as well as for *cna* (collagen-binding protein).

Discussion

Isolates of clonal lineage ST398 seem not to be frequently represented among the *S. aureus* population. They were not recorded by Grundmann et al. (*13*) among a population sample of nasal colonizers in the Nottingham area in the United Kingdom and were not found among 108 isolates from carriers in a rural territory in northern Germany (S. Holtfreter et al., unpub. data). Only 2 notations of ST398 are found in the *S. aureus* MLST database (www.mlst.net), 1 from the Netherlands and 1 from the Cape Verde Islands.

Among 11,250 isolates of various origin (colonization and infections in hospitals as well in the community in humans from all Germany) typed from 1992 through 2003, no isolates refractory to *Sma*I macrorestrition analysis were seen. Therefore, a rather recent emergence of MRSA ST398 among humans seems likely. However, MRSA of lineage ST398 had been reported from infections in pigs

Table. Typing characteristics of methicillin-resistant *Staphylococcus aureus* of clonal lineage ST398, Central Europe

Country/area	Carrier; type of infection	No. isolates	*spa*-type	SCC*mec* group	Resistance phenotype*	Resistance genes
Germany						
Lower Saxony†	Dog; skin infection	1	t034	V	PEN, OXA, ERY, CLI, OTE	*mecA*, *ermA*, *tetM*
	Human‡ (n = 4); nasal carriage	1 3	t011 t034	V	PEN, OXA, ERY, CLI, OTE	*mecA*, *ermA*, *tetM*
	Pig; colonization	1	t034	V	PEN, OXA, ERY, CLI, OTE	*mecA*, *ermA*, *tetM*
	Foal; sinusitis	1	t1197	V	PEN, OXA, OTE	*mecA*, *tetM*
Schleswig-Holstein	Human; infection of skin (outpatient)	1	t011		PEN, OXA, OTE	*mecA*, *tetM*
Lower Saxony†	Human; wound infection	1	t011	V	PEN, OXA, OTE, CIP	*mecA*, *tetM*
	Human; nasal colonization§	1	t034	V	PEN, OXA, ERY, CLI, OTE	*mecA*, *ermA*, *tetM*
Hesse	Human; nasal colonization§	1	t034	V	PEN, OXA, OTE	*mecA*, *ermA*, *tetM*
Saxony-Anhalt	Human; ventilator-associated pneumonia	1	t034	V	PEN, OXA, ERY, CLI, OTE	*mecA*, *ermC*, *tetM*
Saxony	Human (n = 2); ventilator-associated nosocomial pneumonia¶	2	t011	V	PEN, OXA, ERY, CLI, OTE, CIP	*mecA*, *ermC*, *tetM*
Baden-Württemberg	Human (n = 4); ventilator-associated nosocomial pneumonia#	4	t011	V	PEN, OXA, GEN, ERY, CLI, OTE	*mecA*, *ermC*, *tetM*
Austria						
Vienna	Horse (n = 2); wound infection	2	t011	IVa	PEN, OXA, GEN, ERY, CLI, OTE	*mecA*, *ermC*, *tetM*, *aph2"-aac6'*

*PEN, penicillin; GEN, gentamicin; ERY, erythromycin; CLI, clindamycin; OTE, oxytetracycline; CIP, ciprofloxacin.
†Two Lower Saxony regions are listed separately because the isolates originated from different locations within the region and, although belonging to the same clonal lineage, exhibited different *spa*-sequence types and resistance traits with regard to *ermA* and erythromycin resistance.
‡Staff members who worked in the practice where the dog was treated.
§Colonization detected upon hospital admission.
¶Infections at the same ward within a 2-week period.
#Infections at the same ward within a 10-day period.

and from nasal colonization in pig farmers in France (*14*). A more recent report from the Netherlands describes MRSA of ST398 (*spa* t108, which is in the same BURP cluster as t11 and t34) in pigs and in humans who had contact with pigs (*15*). A comparison of the allelic profile of ST398 by means of the MLST database does not indicate any relationship to profiles of prevalent clonal complexes of methicillin-susceptible *S. aureus* (*13*), of epidemic healthcare-associated MRSA, or of *lukS-lukF*–containing community-associated MRSA from Europe.

Conclusions

MRSA exhibiting ST398 may colonize and cause infections in humans and in certain animal species such as dogs, horses, and pigs. The isolation of MRSA ST398 showing the same characteristics from a wound infection in a dog and from nasal colonization of the staff of a veterinary practice where this dog had been treated suggests that interspecies transmission may occur. The differences in *spa* types between the isolates containing the same PCR results for SCC*mec* can be explained by a single genetic event. Because isolates taken at the same time from nasal colonization in veterinary staff of the same practice exhibit either *spa*-type t011 or t034, this difference does not justify discrimination between the two types. Of particular concern was the subsequent detection of MRSA ST398 not only in outpatients but also in inpatients with ventilator-associated pneumonia in the same hospital unit at about the same time (Table).

Future recording of MRSA ST398 from infected and colonized humans (especially when detected by screening at admission to hospitals) will require a thorough analysis with respect to association with animals and routes of transmission. Tracing MRSA carriers among contacts should also include pet animals, horses, and other livestock. Because of the time and labor needed to complete MLST, *spa*-typing combined with BURP analysis of types is an efficient tool for recognizing this clonal lineage.

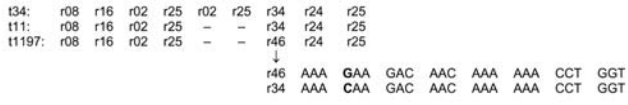

Figure. Repeats of the X-region in methicillin-resistant *Staphylococcus aureus* of clonal lineage ST398.

Furthermore, detection of MRSA by appropriate methods should be implemented into antimicrobial resistance surveillance programs in veterinary medicine.

Prof Witte is head of the laboratory for nosocomial infections at the Robert Koch Institute, Wernigerode Branch. He also is a professor on the medical faculty of Magdeburg University.

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Address for correspondence: Wolfgang Witte, Robert Koch Institute, Wernigerode Br, Burgstraße 37, 38855 Wernigerode, Germany; email: wittew@rki.de

Campylobacter Antimicrobial Drug Resistance among Humans, Broiler Chickens, and Pigs, France

Anne Gallay,* Valérie Prouzet-Mauléon,† Isabelle Kempf,‡ Philippe Lehours,† Leila Labadi,† Christine Camou,† Martine Denis,‡, Henriette de Valk,* Jean-Claude Desenclos,* and Francis Mégraud†

We describe isolates from human *Campylobacter* infection in the French population and the isolates' antimicrobial drug resistance patterns since 1986 and compare the trends with those of isolates from broiler chickens and pigs from 1999 through 2004. Among 5,685 human *Campylobacter* isolates, 76.2% were *C. jejuni*, 17.2% *C. coli*, and 5.0% *C. fetus*. Resistance to nalidixic acid increased from 8.2% in 1990 to 26.3% in 2004 ($p<10^{-3}$), and resistance to ampicillin was high over time. Nalidixic acid resistance was greater for *C. coli* (21.3%) than for *C. jejuni* (14.9%, $p<10^{-3}$). *C. jejuni* resistance to ciprofloxacin in broilers decreased from 31.7% in 2002 to 9.0% in 2004 ($p = 0.02$). The patterns of resistance to quinolones and fluoroquinolones were similar between 1999 and 2004 in human and broiler isolates for *C. jejuni*. These results suggest a potential benefit of a regulation policy limiting use of antimicrobial drugs in food animals.

Campylobacter infections are, along with *Salmonella* infections, the most common cause of bacterial diarrhea in humans worldwide (*1–6*). A recent study on illness and death due to foodborne infections in France estimated an isolation rate of 27–37/100,000 persons/year for *Campylobacter* infection (*7*).

Campylobacter are part of normal enteric flora in animals (poultry, pigs, and cattle) and can be transmitted to humans through contaminated foods (*8*). Several studies identified chicken as the main source of infection (*9,10*). Most *Campylobacter* enteric infections are self-limited and do not require antimicrobial drug treatment. However, severe or long-lasting *Campylobacter* infections do occur and may justify antimicrobial drug therapy. Macrolides as first-line therapy and fluoroquinolones as alternative therapy are recommended (*2,11*). Resistance of *Campylobacter* to antimicrobial agents has increased substantially during the past 2 decades and has become a matter of concern in severe human *Campylobacter* infections (*12–14*). Combined studies in humans and poultry have implicated the use of fluoroquinolones in poultry in the emergence of drug resistance (*15–17*). As a consequence, in 2004 the US Food and Drug Administration withdrew the 1995/1996 approval for the new animal drug application to use enrofloxacin for prophylaxis treatment or growth promotion in poultry (*18*). Veterinary licensing of enrofloxacin in poultry was approved by the European Union (EU) in 1991, and in 1999 the EU recommended limiting the use of fluoroquinolones in poultry.

In this article, we describe characteristics of human *Campylobacter* isolates in France and trends of antimicrobial resistance in such isolates from 1986 to 2004. Trends of *Campylobacter* antimicrobial drug resistance in human isolates were compared with those of isolates from broiler chickens and pigs between 1999 and 2004.

Materials and Methods

National Surveillance System for Human *Campylobacter* Infections

Surveillance for *Campylobacter* infections in France is based on a network of laboratories that send their isolates to the National Reference Center for *Campylobacter* and *Helicobacter* (CNRCH). The network of participating laboratories, limited to hospital laboratories from 1986 to 2001, was complemented by private (which usually cared

*Institut de Veille Sanitaire, Saint Maurice, France; †Centre National de Référence des *Campylobacter* and *Helicobacter*, Bordeaux, France; and ‡Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France

for outpatients) and additional hospital laboratories in 2002 to be more representative of the whole French territory (*19–21*). The network is currently composed of 325 private laboratories (9% of the 3,444 registered private laboratories in France), located in 90 of the 95 districts in mainland France, and 92 hospital laboratories (25% of the 409 registered hospital laboratories). Participating laboratories perform a systematic screening for *Campylobacter* in stools. Each isolate recovered is sent to CNRCH in a transport medium (medium for storage of bacteria, Bio-Rad, Marnes-la-Coquette, France) with information on the type of specimen; date and district of isolation; patient's age, sex, and history of travel abroad; and eventual context of an outbreak.

On reception at CNRCH, isolates are tested for viability, confirmed as *Campylobacter* by standard phenotypic identification, and identified at the species level with phenotypic methods and real-time PCR to differentiate between *C. jejuni*, *C. coli* and *C. fetus* (*2*,*22*). The other species are identified by comparing their 16S rDNA sequences to those of DNA databases by using the BLAST program (*23*). Identification at the species level is considered correct when at least 99% identity occurs with only 1 species.

Antimicrobial Drug Resistance Monitoring of *Campylobacter* in Humans

Campylobacter isolates from all species were evaluated for susceptibility to 7 antimicrobial drugs (nalidixic acid, ciprofloxacin [since 2000], erythromycin, amoxicillin, gentamicin, tetracycline, and doxycycline [since 2003]) by the agar diffusion method on Mueller-Hinton agar enriched with 5% sheep blood by using antibiotic disks, according to recommendations for *Campylobacter* of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (*24*). Since the hospital laboratory network set up in 1986 was extended to private laboratories in 2002, antimicrobial susceptibility trends were analyzed exclusively for hospital laboratory isolates between 1986 and 2004. Multidrug resistance was defined as resistance to $\geq$2 antimicrobial drugs.

Antimicrobial Drug Resistance Monitoring in Broilers and Pigs

Surveillance of *Campylobacter* antimicrobial drug resistance was implemented in France in 1999 for broilers in conventional and free-range broiler farms and in 2000 for pigs as part of a surveillance program on resistance in sentinel bacteria (*Escherichia coli* and *Enterococcus* spp.) and zoonotic bacteria (*Salmonella* spp. and *Campylobacter* spp.) in animal products for human consumption. Thus, data collection began just after the ban of 4 antimicrobial growth promoters (bacitracin zinc, spiramycin, virginiamycin, and tylosin phosphate) by the European Community (EC) Council Regulation (No. 2821/98, December 1998). Conventional broiler flocks are characterized by an indoor rearing period of 6 weeks, and free-range broiler flocks have an indoor rearing period of 6 weeks followed by 6 additional weeks with access to an open-air area.

From 200 to 600 broiler cecal samples or pig fecal samples were collected each year in 10 broiler and 10 pig slaughterhouses representative of French production of these animals for human consumption (*25*). Strain isolation was performed in a central laboratory (Agence Française de Sécurité Sanitaire des Aliments [AFSSA], Ploufragan, France) for the first 2 years and then in district veterinary laboratories, except for antimicrobial susceptibility testing. After identifying isolates by using multiplex PCR (*26*), the MIC of ampicillin, nalidixic acid, enrofloxacin or ciprofloxacin, tetracycline, erythromycin, and gentamicin were determined by agar dilution. As for human isolates, susceptibility to antimicrobial drugs was categorized according to the 2004 statement of the CA-SFM (*24*). The study of antimicrobial resistance of *Campylobacter* from animal sources was supported by the French Ministry of Agriculture.

Statistical Analysis

Differences between proportions and isolation rates were tested by χ^2 and Fisher exact tests. Means were compared with Student and Fisher tests. Patterns of antimicrobial resistance were analyzed by 4-year increments from 1986 to 2004.

Results

Surveillance for Human *Campylobacter* Infections

From April 2002 to December 2004, CNRCH received 5,685 presumptive *Campylobacter* isolates. Among the 5,112 (89.9%) viable isolates, 3,896 (76.2%) were *C. jejuni*, 878 (17.2%) *C. coli*, 257 (5.0%) *C. fetus*, 21 (0.4%) *C. lari*, 40 (0.8%) *Arcobacter butzleri*, and 13 (0.25%) other species of *Campylobacter.* Seven strains (0.1%) were *Helicobacter* spp. A seasonal increase during the warmer months was noted and was more pronounced for *C. jejuni*.

The median age of patients was 29.4 years (range 5 days–100 years). Thirteen (0.2%) were newborns (5–30 days), 258 (4.5%) infants (1–11 months), 1,907 (33.5%) children (1–10 years), 2,555 (44.9%) ages 11–65 years, and 767 (13.5%) >65 years (Figure 1). Isolation of *Campylobacter* was more frequent among male than female patients (male/female ratio = 1.2, p = 0.04), except for young adults (16–30 years), with a male/female ratio = 0.9 ($p<10^{-3}$). The ratio of *C. jejuni* to *C. coli* varied between

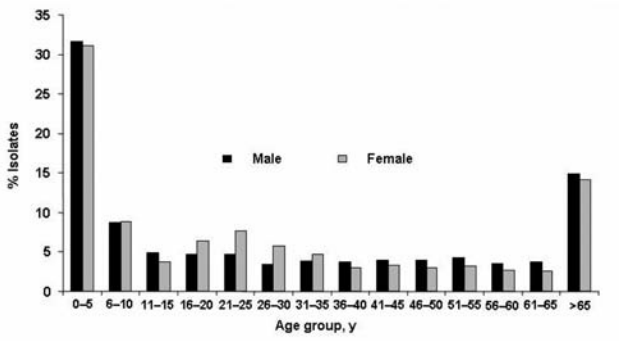

Figure 1. Distribution of *Campylobacter* isolates according to age and sex of patient, France, 2002–2004.

4.5 and 7.2 in those <30 years of age and decreased thereafter. *C. fetus* was isolated among adults >30 years of age and peaked in the elderly ($p<10^{-3}$, Figure 2).

Among the 5,620 isolates with a known clinical source, 5,253 (93.4%) were isolated from stools, 308 (5.5%) from blood, and 50 (0.9%) from other sites presumably seeded as a result of bacteremic infections. Both *C. jejuni* and *C. coli* were isolated essentially from stools, whereas 158 (63.5%) of 249 *C. fetus* isolates were from blood. Patients with blood isolates were older than those with stool isolates (median age 69 years vs. 19.3 years, $p<10^{-3}$).

Travel history was available for 1,370 (24.1%) case-patients; 184 (3.2%) reported traveling outside France during the 2 weeks before onset of illness. The country of travel was specified for 169 (91.8%) case-patients (Africa, 98 persons; Asia, 26; Europe, 16; South America, 10; and other countries, 19).

Antimicrobial Drug Resistance of Human *Campylobacter* Isolates

Resistance to nalidixic acid and tetracycline/doxycycline increased from 1986–1989 to 2002–2004 ($p<10^{-3}$, Figure 3). Resistance to ampicillin, although frequent, decreased from 49.2% (1,027/2,087) in 1986–1989 to 42.4% (501/1,198) in 2002–2004 ($p<10^{-3}$). Resistance to erythromycin remained low, and no isolate was resistant to gentamicin.

Nalidixic acid resistance increased from 8.2% (26/315) in 1990 to 26.3% (115/438) in 2004 ($p<10^{-3}$). Resistance was greater for *C. coli* (21.3%) than *C. jejuni* (14.9%, $p<10^{-3}$, Figure 4). Nalidixic acid resistance increased for *C. jejuni* >4-fold from 1995 to 1997 and for *C. coli* >3-fold from 1994 to 1996. Then, resistance decreased for both *C. coli* and *C. jejuni* in 1999 but remained higher than before 1995 (Figure 4). Ciprofloxacin resistance, tested since 2000, followed the same pattern (Table).

Fifty-eight percent of *Campylobacter* isolates were resistant to ≥1 drug, 34.7% to ≥2 drugs, and 20.0% to ≥3 drugs. The most common multidrug resistance (≥2 drugs) patterns included resistance to nalidixic acid or ciprofloxacin, to doxycycline, and to ampicillin.

Among the case-patients ≤15 years of age, 28.0% (618/2,207) had a *Campylobacter* strain resistant to nalidixic acid compared with 37.6% (1,029/2,736) of the case-patients >15 years of age ($p<10^{-3}$). The proportion of resistance to ciprofloxacin did not vary according to age (27.3% of case-patients ≤15 years and 27.9% >15 years). For ampicillin, 41.9% (925/2,207) of case-patients ≤15 years had a resistant strain compared with 37.3% (1,024/2,736) of the case-patients >15 years ($p = 0.001$).

Of the case-patients who traveled abroad, for which strain resistance was available, 40.3% (67/166) had a strain resistant to ciprofloxacin, compared with 27.0% (294/1,090) of case-patients who did not travel abroad ($p<10^{-3}$). For nalidixic acid, 42% (70/166) of case-patients who traveled abroad compared with 34.7% (378/1,090) of case-patients who did not had a resistant strain ($p = 0.06$). Resistance to ampicillin was present for 28.3% (47/166) who had traveled abroad compared with 31.1% (339/1,090) for those who had not ($p = 0.01$).

Antimicrobial Resistance in Broilers and Pigs

Between 1999 and 2004, a total of 544 *C. jejuni* and 374 *C. coli* isolates were recovered from poultry, and 871 *C. coli* were recovered from pigs by the antibiotic resistance surveillance system. Among the broiler isolates, the proportion of *C. jejuni* from animals raised in standard and export production facilities gradually decreased from 83.5% (279/334) in 1999 to 43% (28/65) in 2004 ($p<10^{-3}$), while the proportion of *C. jejuni* decreased from 32% (18/57) to 10% (4/40) in the free-range production facilities ($p = 0.01$).

Campylobacter isolates were inconstantly sensitive to ampicillin, and a high proportion of isolates resistant to tetracycline was recorded in poultry and pigs, but all strains remained sensitive to gentamicin (Table). Isolates from pigs were less frequently resistant to ampicillin but more often resistant to tetracycline. For erythromycin,

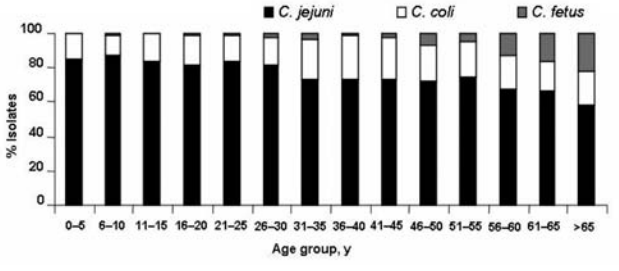

Figure 2. Distribution of human *Campylobacter* strains by species and patient age group, France, 2002–2004.

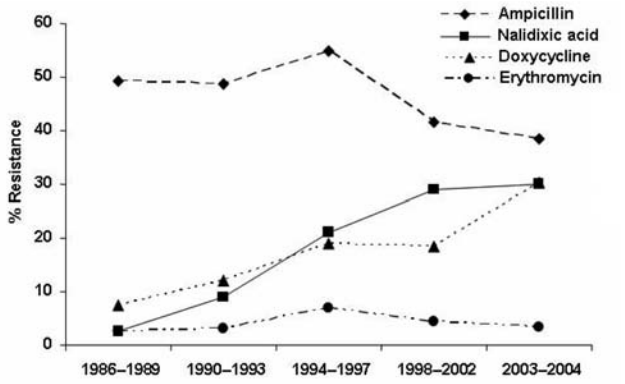

Figure 3. Resistance of human *Campylobacter* spp. isolates to antimicrobial agents, France, 1986-2004.

resistance was rare among *C. jejuni* strains (1.8%), but much more frequent for *C. coli* (21.1% of broiler isolates and 58.9% of pig isolates, $p<10^{-3}$).

In broilers, *C. coli* strains were more often resistant to ciprofloxacin (37.4%, 140/374) than were *C. jejuni* strains (19.5%, 106/544), $p<10^{-3}$) (Table, Figure 5). *C. coli* resistance to ciprofloxacin increased in pigs from 12.3% (39/316) in 2000 to 26.9% (18/67) in 2004 ($p = 0.002$). For *C. jejuni* in broilers, after an increase in resistance from 16.8% (50/297) in 1999 to 31.7% (13/41) in 2002 ($p = 0.02$), resistance to ciprofloxacin decreased to 9.0% (3/32) in 2004 ($p = 0.02$) (Table, Figure 5). Similar trends were observed for quinolone resistance. The trend of resistance to nalidixic acid and ciprofloxacin was similar for *C. jejuni* isolated from humans and broilers between 1999 and 2004 (Table, Figure 5).

Discussion

Our surveillance of *Campylobacter* isolates in France indicates some differences with findings from other western countries, i.e., a greater proportion of *C. coli* (17.0%). The epidemiologic characteristics of *Campylobacter* infections were, however, similar. *Campylobacter* is predominant in the summer (*27*), the isolation rate was much greater in children <5 years of age, and *C. jejuni* was predominant. The rate of resistance to certain antimicrobial drugs increased substantially from 1990 to 2004, and the proportion of resistant isolates varied according to *Campylobacter* sp. Resistance to quinolones was greater for *C. coli* (21.3%) than for *C. jejuni* (14.9%). Quinolone and fluoroquinolone resistance for *C. coli* was high in broilers and pigs. Resistance to quinolone and fluoroquinolone for *C. jejuni* had the same pattern over time in broilers and human isolates.

The proportion of *C. coli* was higher in France (17%) than in the United States (<1%) or Belgium (11%) (*2*,*28*). Methods for characterization of the species vary by country and by laboratory within a country (*29*,*30*). In France, CNRCH routinely characterizes the species using a combination of phenotypic and molecular methods (specific PCR) with verification of discrepant results (*22*). In some other countries, *Campylobacter* are not routinely characterized at the species level and could be incorrectly identified as *C. jejuni* or *Campylobacter* spp. This may account for an underestimation of species other than *C. jejuni* in some countries and therefore some distortion of the proportion of antimicrobial drug resistance by species. In France, the high proportion of *C. coli* isolates is probably real, as an increasing proportion of *C. coli* is colonizing broilers (*31*). This trend may be related to the use of different isolation and identification methods, to a recent increase in the ratio of *C. coli* to *C. jejuni*, or both. The ban of antimicrobial growth promoters and of animal protein–based feed may have influenced the digestive bacterial flora equilibrium of chickens. Udayamputhoor et al. showed that the ceca of birds that receive plant protein–based feed are less likely to be colonized with *C. jejuni* than the ceca of birds that receive other types of feed (*32*). Another hypothesis is that because 100% of pigs are colonized by *C. coli* in France, the proximity of pig and poultry farms in the main producing regions may result in cross-contamination. However, this explanation is unlikely because *C. coli* strains isolated from broilers and pigs had different antimicrobial resistance patterns, and *C. coli* poultry strains clustered separately from those of porcine origin (*33*). Nonetheless, strains may undergo different selection pressures.

Resistance to ampicillin is of clinical interest because this drug may be used for the treatment of severe *Campylobacter* infections. The proportion of resistance to ampicillin was higher among patients who did not travel than among patients who did and in children ≤15 years. In addition, resistance of *Campylobacter* isolates in humans did not follow the same patterns over time as resistance in broiler and porcine isolates. These results suggest that resistance to ampicillin is more frequently domestically acquired and may be related to the use of ampicillin in human therapy because ampicillin is widely prescribed for infections in children.

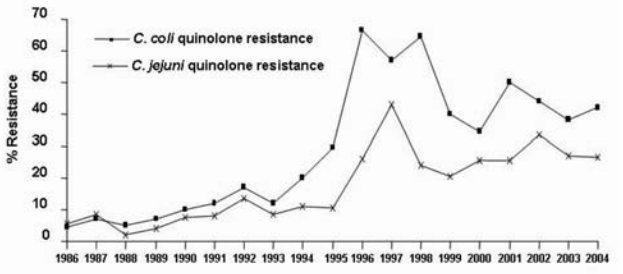

Figure 4. Quinolone resistance of human *Campylobacter jejuni* and *C. coli*; France, 1986–2004.

Table. Resistance of *Campylobacter jejuni* and *C. coli* in humans, broilers, and pigs to ampicillin, erythromycin, tetracycline/doxycycline, and ciprofloxacin/enrofloxacin, France, 1999–2004

	C. jejuni		*C. coli*		
Antimicrobial agent/year	Humans, no. (%)	Broilers, no. (%)	Humans, no. (%)	Broilers, no. (%)	Pigs, no. (%)
Ampicillin					
1999	123 (42.3)	297 (22.6)	20 (70.0)	96 (29.2)	*
2000	189 (40.2)	67 (31.3)	35 (37.1)	35 (31.4)	317 (12.3)
2001	137 (47.4)	61 (13.1)	26 (53.8)	44 (25.0)	291 (11.0)
2002	184 (45.6)	41 (29.3)	43 (48.8)	64 (28.1)	101 (14.9)
2003	479 (46.1)	46 (34.8)	68 (28.1)	60 (30.0)	101 (17.8)
2004	438 (38.1)	32 (28.1)	88 (14.9)	74 (24.3)	67 (7.5)
Erythromycin					
1999	123 (0.8)	297 (0.3)	20 (10.0)	96 (31.3)	*
2000	187 (3.2)	67 (3.0)	35 (8.6)	36 (11.1)	317 (65.3)
2001	136 (3.7)	61 (4.9)	26 (7.7)	44 (4.5)	289 (49.1)
2002	184 (2.7)	40 (5.0)	44 (9.0)	64 (17.2)	101 (58.4)
2003	478 (0.6)	46 (4.3)	68 (7.3)	61 (31.1)	97 (78.4)
2004	437 (1.4)	32 (0.0)	88 (12.5)	74 (17.6)	67 (43.3)
Tetracycline/doxycycline					
1999	118 (23.7)	297 (56.6)	6 (25.0)	96 (69.8)	*
2000	188 (12.8)	67 (55.2)	35 (14.3)	35 (60.0)	317 (82.6)
2001	137 (9.5)	61 (65.6)	26 (26.9)	45 (80.0)	289 (88.9)
2002	184 (22.8)	41 (67.5)	43 (41.8)	64 (84.4)	101 (86.1)
2003	479 (26.7)	46 (60.9)	68 (63.2)	61 (96.7)	97 (95.9)
2004	438 (28.8)	32 (40.6)	88 (53.4)	74 (71.6)	67 (61.2)
Ciprofloxacin/enrofloxacin					
1999	†	297 (16.8)	†	96 (39.6)	*
2000	185 (23.8)	68 (23.5)	35 (31.4)	35 (28.6)	316 (12.3)
2001	137 (21.9)	61 (29.5)	26 (34.6)	45 (37.8)	292 (12.3)
2002	184 (31.7)	41 (31.7)	44 (43.2)	63 (41.3)	101 (21.8)
2003	479 (25.9)	45 (13.3)	68 (38.2)	61 (41.0)	99 (24.2)
2004	438 (25.3)	32 (9.4)	88 (42.0)	74 (32.4)	67 (26.9)

*Strains isolated from poultry in 1999 and 2000 were tested with enrofloxacin; strains isolated between 2001 and 2004 were tested with ciprofloxacin; all pig strains were tested with ciprofloxacin.
†Human strains have been tested for ciprofloxacin resistance since 2000.

Nalidixic acid resistance increased 5-fold from 1990 (5.3%) to 2004 (26.3%), consistent with trends observed in other countries (*16*,*17*). The use of fluoroquinolones in animal feed was approved in Europe in 1990. Studies have shown the development of ciprofloxacin-resistant *Campylobacter* in treated chickens and the spread of ciprofloxacin-resistant *Campylobacter* from animal food sources to humans (*17*,*34*,*35*). Australia, where fluoroquinolones have never been licensed for use in food-producing animals, did not experience fluoroquinolone resistance in human *Campylobacter* isolates (*36*).

The high proportions of resistance to nalidixic acid and ciprofloxacin in broilers and pigs are consistent with the findings of Desmonts et al. in France (*31*). In this study, quinolone and fluoroquinolone resistance increased between 1992–1996 and 2001–2002 among isolates from standard chicken flocks, while resistance remained low for free-range flock isolates. In France, antimicrobial growth promoters have never been authorized in the production of free-range chickens, contrary to standard methods of production of chicken flocks, and antimicrobial therapy is limited (*31*).

From 2002 to 2004, ciprofloxacin resistance dropped substantially in *C. jejuni* isolated from broilers; nalidixic acid resistance decreased as well, although not significantly. The decrease in broilers may be related to the restriction in the use of fluoroquinolones in animal feed after the 1999 EU recommendation. Similarly, in Denmark, resistance to macrolides of *C. coli* declined after the prophylactic and growth-promoting use of macrolides was banned (*37*). However, the decrease in ciprofloxacin resistance occurred 2–3 years after the EU recommendation, which suggests that EU recommendations were not followed immediately by application or, alternatively, that the effect of the restriction in the use of fluoroquinolones in animal feed is not immediate. Unfortunately, no resistance data in broilers and pigs were available before 1999, which is a limitation to interpret recent trends in relation to the EU recommendations. According to the French food security agency (AFSSA), global sales of antimicrobial agents decreased consistently from 2001 through 2002, but information on species-specific consumption was not available (*38*). Specific data from veterinary prescriptions and livestock consumption

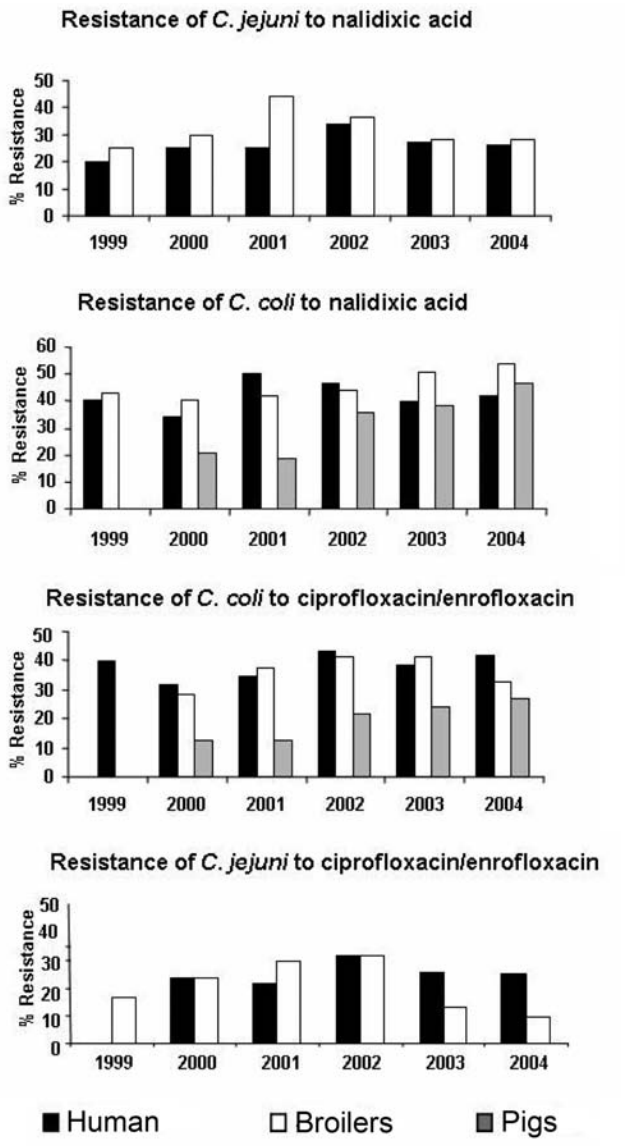

Figure 5. Resistance of *Campylobacter jejuni* and *C. coli* to nalidixic acid and ciprofloxacin in humans, broilers, and pigs, France, 1999–2004. Human strains were tested with ciprofloxacin since 2000. Strains isolated from poultry and pigs in 1999 and 2000 were tested with enrofloxacin, strains isolated between 2001 and 2004 were tested with ciprofloxacin.

are necessary to quantify the amount of antimicrobial agents consumed by animals.

C. jejuni nalidixic acid and ciprofloxacin resistance decreased concomitantly in humans and broilers from 2002 to 2004. Because the decrease was less pronounced in humans than in broilers, a longer period is needed to detect an effect of the restriction in the use of antimicrobial agents in animal feeds or resistance may be also related to other exposure. Fluoroquinolones are the first drugs of choice for the empiric treatment of human diarrhea or prophylactic treatment associated with travel in France and may be responsible for a part of resistance in humans (*39*). However, >80% of patients infected with a ciprofloxacin-resistant strain did not travel to a foreign country before onset of illness, which indicates that a substantial proportion of fluoroquinolone resistance was domestically acquired (*40*). The resistance rate to ciprofloxacin was not higher in adults compared with children, as could be expected if treatment of cases was contributing to resistance (*39*), because fluoroquinolone treatment is not used in children ≤15 years of age.

In contrast to *C. jejuni*, we observed no decrease in quinolone and fluoroquinolone resistance in *C. coli* in pigs, broilers, or human isolates. The use of these antimicrobial agents in pigs may not have changed, and a part of human *C. coli* infection may be related to other sources. Alternatively, unknown mechanisms could be implicated in *C. coli* resistance, such as a high number of point mutations.

Our study has several limitations. Laboratories participate voluntarily in the surveillance network, which may result in a selection of strains that is not representative of the general population. However, all districts of mainland France were included. In addition, this limitation, if it exists, may not affect antimicrobial drug resistance because participating laboratories routinely screen stools for *Campylobacter* and send all their isolates to CNRCH. Since the laboratory network was extended to private and additional hospitals laboratories in 2002, this may have had an effect on the trends in resistance. However, trends of human antimicrobial susceptibility were analyzed exclusively among strains from hospital laboratories from 1986 to 2004. In addition, characteristics of *Campylobacter* isolates sent to CNRCH did not change for age, sex, seasonality, and species after the network extension in 2002 (*20*,*21*). Comparison of human and animal data was not based on a continuum between human isolates and contaminated food consumption (isolates from retail chicken). However, broiler chicken and pig data were representative of French livestock and were consistent with those of another recent survey done in France (*31*).

The extension of the surveillance of human *Campylobacter* allowed the epidemiologic characteristics of *Campylobacter* infections that occurred in the general French population to be better understood. *Campylobacter* resistance to antimicrobial agents increased to a high level among humans in France from 1990 through 2004. Comparison of antimicrobial resistance patterns in humans, broilers, and pigs from 1999 to 2004 showed similar patterns of quinolone and fluoroquinolone resistance for *C. jejuni* isolates from broilers and humans. These results suggest that a limitation of the use of fluoro-

quinolones in broilers may reduce fluoroquinolone resistance of *Campylobacter* in humans. Other studies, however, are needed to further quantify the effect of restricted use of antimicrobial drugs in animals on bacterial resistance in human isolates. Ongoing national surveillance of *Campylobacter* in humans, livestock, and animal feeds at the retail level and antimicrobial susceptibility testing are necessary to evaluate the effects of implementing European policies. Further research is also needed to better understand the relationship between antimicrobial use in animals and humans and bacterial resistance in humans.

Acknowledgments

We thank the private community and public hospital laboratories that participated in the national surveillance of *Campylobacter* infections in humans by sending their isolates to CNRCH.

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Dr Gallay is an epidemiologist in the unit of foodborne diseases at the Institut de Veille Sanitaire, Saint Maurice, France. She directs the program on *Campylobacter* infections and foodborne viruses in France. Her research interests also include foodborne and waterborne outbreaks.

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Address for correspondence: Anne Gallay, Institut de Veille Sanitaire, Département des maladies infectieuses 12, rue du Val d'Osne 94 415 Saint Maurice, France; email: a.gallay@invs.sante.fr

Host-associated Genetic Import in *Campylobacter jejuni*

Noel D. McCarthy,* Frances M. Colles,* Kate E. Dingle,* Mary C. Bagnall,† Georgina Manning,† Martin C.J. Maiden,* and Daniel Falush*

Host association of *Campylobacter jejuni* was analyzed by using multilocus sequence typing data for 713 isolates from chickens and bovids (cattle and sheep). Commonly used summary measures of genotypes (sequence type and clonal complex) showed poor accuracy, but a method using the full allelic profile showed 80% accuracy in distinguishing isolates from these 2 host groups. We explored the biologic basis of more accurate results with allelic profiles. Strains isolated from specific hosts have imported a substantial number of alleles while circulating in those host species. These results imply that 1) although *Campylobacter* moves frequently between hosts, most transmission is within species, and 2) lineages can acquire a host signature and potentially adapt to the host through recombination. Assignment using this signature enables improved prediction of source for pathogens that undergo frequent genetic recombination.

Many human pathogens inhabit several animal host and environmental reservoirs, and a broad host range is particularly characteristic of emerging diseases (*1*). Identification of the relative contributions of pathogen sources and transmission routes is necessary to support evidence-based disease control programs (*2*). One approach to this identification, microbial source tracking, is the application of microbial typing to isolates from human cases and possible sources in the food chain to enable attribution of disease to food sources at individual case and population levels (*3*,*4*). Evidence-based control programs using this information have worked well for *Salmonella* at a population level in Denmark (*4*).

Source tracking depends on accurate estimation of the frequency of different subtypes in each host reservoir. For *Salmonella*, specific serotypes and phage subtypes are stably found in the same host (*3*). The biology underlying this is first that specific clones are well-adapted to specific hosts and second that the combination of serotype and phage type provides a stable and reliable indicator of a specific clone. For other organisms it can be difficult to find reliable host-associated markers. One example is *Campylobacter jejuni*, the most common bacterial zoonosis and the main cause of bacterial gastroenteritis in the western world. Phenotyping has not worked well in determining source. Genetic methods of discrimination show large diversity of results within this species; studies typically report ≈50% as many genotypes as strains (*5*–*12*). Many common genotypes are broadly distributed and it is not possible to estimate the relative frequency of rare genotypes in different host reservoirs accurately. Because of these difficulties, although host associations have been identified for particular genotypes, no generally useable approach has been developed.

We developed an approach that uses multilocus sequence typing (MLST) data to identify the reservoir of origin of a strain. This approach was tested by using isolates from known sources (cattle, sheep, and chickens), which allowed us to compare our predictions with the true origin of each strain. This method can provide reasonably accurate results for rare or unique genotypes and for clones that are broadly distributed. This approach takes into account frequent recombination in *Campylobacter*, which limits the accuracy of approaches based on the *Salmonella* paradigm.

Methods

Data

MLST of *C. jejuni* is based on sequencing 7 loci with lengths of 402–507 bp separated from each other by ≥15,000 bp in the type strain (*10*). We used MLST data in 3 different forms. The first form was the sequence type (ST), which is a unique combination of 7 alleles. STs index

*University of Oxford, Oxford, United Kingdom; and †Veterinary Laboratories Agency, Weybridge, United Kingdom

the full discrimination available within MLST. The second form was the clonal complex, which is a group of closely related STs, e.g., differing at ≤2 of the 7 alleles. Clonal complexes, if accurately inferred, are groups of strains that share a more recent common ancestor than with strains outside the complex but are not identical to each other at all of the MLST loci (*10*,*13*,*14*). The third form was the 7 allele fragments; we assumed that they each provided independent information.

We included all *C. jejuni* isolates from cattle, sheep and chickens that were in the pubmlst database (www.pubmlst.org) with a date before August 1, 2004, and which had been published in peer-reviewed literature or for which permission to use in this study was obtained from those who had submitted the data. All but 10 of the isolates on pubmlst were available for inclusion by these criteria. We also included additional typed isolates (n = 27) provided by researchers when they were contacted for permission to include unpublished isolates from the pubmlst database. *C. jejuni* has been shown to recombine with *C. coli* (*15*). Those isolates with ≥4 of 7 alleles typical of *C. jejuni* were included. A total of 713 isolates were available by these criteria and came from animal feces, live animals, and dead animal tissue. The distribution of the data by host type and by year and country of isolation is shown in Tables 1 and 2.

Table 1. *Campylobacter jejuni* isolates by year of isolation and host species

Year	Chickens	Cattle	Sheep	Total
1981	0	4	0	4
1982	2	1	2	5
1983	0	3	0	3
1984	0	1	0	1
1986	0	2	0	2
1988	2	18	0	20
1989	0	1	0	1
1990	54	1	0	55
1991	30	6	0	36
1992	1	3	0	4
1993	8	6	1	15
1994	6	1	0	7
1995	12	1	0	13
1996	35	0	0	35
1997	2	0	0	2
1998	40	41	68	149
1999	10	38	38	86
2000	15	6	0	21
2001	45	83	5	133
2002	0	0	2	2
2003	13	0	0	13
Unspecified	34	29	43	106
Total	309	245	159	713

Population Assignment

Differences in genotype frequency between populations enable probabilistic assignment of isolates to populations, even if some sharing of genotypes occurs between those populations. We used STRUCTURE, a model-based clustering method designed to infer population structure and assign individuals to populations using multilocus genotype data (*16*). The source of the isolates to be assigned was predicted on the basis of a training set that consisted of other relevant isolates. In order to do this predicting, we used the USEPOPINFO option, which allows the population of origin to be known for some strains (in this instance, the training set) while for other strains (the isolates to be assigned) this population is assumed unknown.

STRUCTURE estimates the genotype frequencies in each host species based on all of the isolates; it also estimates the population of origin for isolates of unknown origin, taking into account uncertainty due to sample size. To enable maximum use of data, some analyses used a leave-one-out strategy in which 1 isolate was assigned by using the remaining strains as the training dataset and the procedure was repeated for each isolate.

The parameters we used for all STRUCTURE simulations were a no-admixture model with $\lambda = 1$ and gene frequencies uncorrelated between populations. We ran 1,000 burn-in cycles and 10,000 additional repetitions for each analysis. Empiric assignment accuracy was measured as the average probability pk^* with which each isolate was assigned to the correct host source k^*. Predicted assignment accuracy is estimated as the average of

$$\sum_{k=1..K} p_k^2 ,$$

where each individual is assigned to 1 of K different sources. The permutation test (Figure, panel A) was performed by randomly permuting the actual host species among the predictions obtained from STRUCTURE repeated 10,000 times.

Results

Among 713 isolates, 330 MLST genotypes were identified. Two isolates (ST-284 and ST-327) had 4 alleles typical of *C. jejuni* and 3 typical of *C. coli*. All others had

Table 2. *Campylobacter jejuni* isolates by country and host species

Country	Chicken	Cattle	Sheep	Total
Canada	0	5	0	5
Czech Republic	8	0	0	8
Denmark	6	1	0	7
The Netherlands	53	4	0	57
New Zealand	5	1	0	6
Northern Ireland	1	2	0	3
United Kingdom	217	218	158	593
United States	17	13	1	31
Unknown	2	1	0	3
Total	309	245	159	713

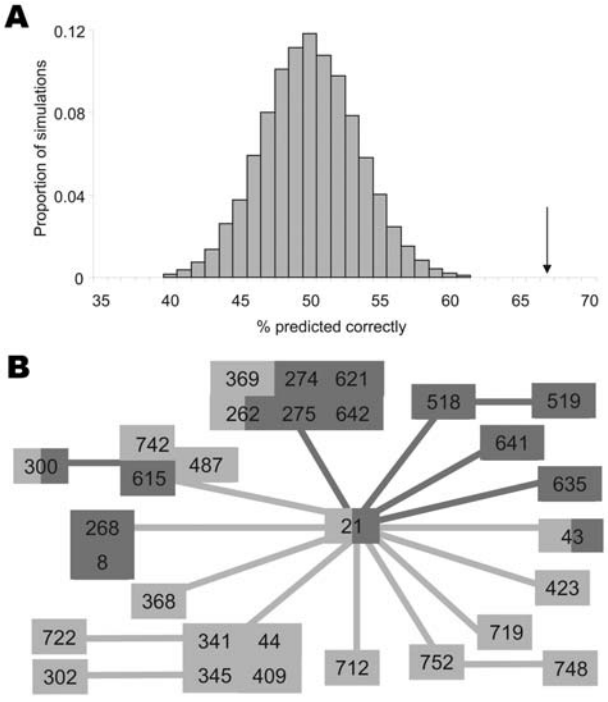

Figure. Prediction of source of origin within the ST-21 complex. A) Observed accuracy of prediction (arrow) compared with distribution of values obtained by permuting host labels so that the alleles varying from central genotype are not informative on host of origin. B) Prediction of origin by using only alleles for which substantial reference information is available. Light lines indicate alleles different from ST-21 present mainly in chickens in the reference population (i.e., an allele that would predict chicken origin); dark lines indicate alleles present mainly in bovids (i.e., predicts bovid origin). Light boxes indicate STs found only in chickens, dark boxes indicate STs found only in bovids, and boxes with light and dark shading indicate STs found in bovids and chickens.

≥5 typical *C. jejuni* alleles. Table 3 shows assignment accuracy when we used the whole dataset and a leave-one-out strategy to assign strains to 3 host species (cow, sheep, and chicken) on the basis of 7 alleles, the clonal complex, the ST, and combinations thereof. Because random assignment would be correct one third of the time, how much improvement genotype information showed compared with random assignment is more informative than the percentage correct, i.e., what proportion of the gap between 33% correct expected by using random assignment and 100% correct with perfect prediction has been closed. Assignment by using the 7 alleles closed 37% of this gap compared with 10% for ST and 13% for clonal complex. Prediction did not improve substantially when ST or clonal complex information was added to allele information. These overall results emphasize the limits in using an ST or clonal complex as a summary of MLST when predicting host of origin. We therefore used alleles in all further analyses and explored the basis for the better accuracy of this approach.

Prediction of host of origin to 3 host sources on the basis of alleles is shown in Table 4. The method showed higher accuracy for distinguishing chicken strains from cow or sheep strains than for distinguishing between strains from the 2 bovid species. When we performed analysis restricted to cattle and sheep isolates, we obtained an assignment accuracy of 58% compared with 50% expected by chance and thus explained only 16% of remaining uncertainty. This additional analysis showed little detectable host association for these 2 closely related host species. Further comparison of chicken isolates with a combined population from cattle and sheep showed improved resolution and allowed correct prediction 80% of the time (60% of uncertainty removed), which indicated substantial host association.

Given the nature of the dataset, we must consider possible confounding factors such as differences in time or location of sampling, which may lead either to completely spurious associations or to overestimates of their magnitude. Indeed, there was evidence for modest time and geographic effects within our dataset. For example, in a comparison of UK chicken isolates in 1997 or earlier and in 1998 or later (Table 5), 66% could be assigned to the population of the correct period based on allelic profile. Similarly, when UK and Dutch chicken isolates were considered, 69% were assigned to the correct country. We therefore performed additional analyses in which host was negatively associated with time, space, or both (Table 5). Late UK chicken isolates (1998–2003) were assigned by

Table 3. Capacity of alleles, overall sequence type, and clonal complex information to predict host species for *Campylobacter jejuni* isolates from cattle, sheep, and chickens

Genotype information used	% Correct	% Uncertainty removed*
Alleles	58	37
Sequence type	40	10
Clonal complex (1)†	42	13
Clonal complex (2)†	42	13
Alleles plus sequence type	60	40
Alleles plus clonal complex†	58	37

*Random selection would be expected to predict correctly 33% of the time. The proportion of the remaining uncertainty (67%) that is resolved is shown.
†Clonal complex (1) substituted sequence type for clonal complex where no clonal complex is assigned and clonal complex (2) substituted a missing value code. Clonal complex (1) was also used to assess alleles plus clonal complex.

Table 4. Comparison of actual host and predicted host among *Campylobacter jejuni* from cattle, sheep, and chickens

Actual host	Sample size (n)	Predicted host, %		
		Chicken	Cow	Sheep
Chicken	309	66	14	19
Cow	245	12	50	38
Sheep	159	10	36	54

using early UK chicken (1997 or earlier) and late UK bovid isolates (1998–2003) as training sets, giving 77% assignment to chickens. UK chicken isolates were assigned by using non-UK chickens and UK bovid isolates as training sets, producing 64% assignment to chickens. These analyses showed that host effect is stronger than that of time or space and that our findings are not the result of confounding by these factors.

To explore the mechanism underlying the better performance observed for allele-based assignment and to better understand the biologic processes that produce this host signature in the bacterial genome, we investigated assignment within the ST-21 complex. This clonal complex comprises a substantial proportion of isolates and is highly diverse (*5*,*10*,*17*,*18*). Our sample contained 252 ST-21 complex isolates. Of these, 188 were not ST-21 but differed at 1 to 3 alleles from the central genotype. We assigned these 188 isolates to chicken or bovid hosts on the basis of alleles at which they differed from ST-21 by using all non–ST-21 complex isolates as the training set. A total of 66% of isolates were assigned to the correct host. This finding suggests that ST-21 complex isolates acquire alleles that are characteristic of the host population. To demonstrate that this deviation from 50% is not a sampling artifact or chance effect, we restricted analysis to the 88 unique ST-host combinations, which largely eliminates the possible effects of clonal expansion within host, and performed a permutation test to assess the possible role of chance. Of these combinations, 67% were correctly assigned, which was a higher proportion than observed in any of 10,000 iterations in a permutation test (Figure, panel A).

The overall accuracy of host assignment based on acquired alleles is limited because many of these alleles are each too rare to enable accurate estimation of their frequency in particular host gene pools. Acquired alleles that are frequently observed give more accurate host prediction. To illustrate this visually (Figure, panel B), we used as predictors only those alleles that are found in ≥10 different ST-host combinations in the non–ST-21 complex isolates and are also substantially differentiated between chicken and bovid populations (on the basis of a 65% cutoff value). All 4 isolates with 2 alleles, both suggestive of either chicken or bovid origin, were from the predicted source. In 1 instance, 2 potentially informative alleles gave conflicting information; 1 suggested bovid origin and 1 suggested chicken origin. Isolates with this ST came from both sources. Of the 24 STs with only 1 informative allele, 18 were correctly assigned; only 4 were incorrectly assigned. The remaining 2 STs were isolated from chicken and bovid sources.

Discussion

Our analyses confirm the association of *C. jejuni* genotypes with host species, and demonstrate a clear distinction between isolates obtained from chickens and those obtained from bovids, when alleles are considered independently in statistical analysis. This finding was robust to sampling differences in time and place and suggested that host effects were stronger than geographic and temporal effects, which must be considered if these associations are to be used in epidemiologic investigations. Moreover, because populations of *C. jejuni* in farm animals such as bovids and chickens may show greater similarity than those from other hosts (*5*,*9*), the approach may be more accurate when considering *C. jejuni* from a more diverse host range. The distinction between cow and sheep isolates is much weaker. Differentiation between these species might be demonstrable if more genetic information was available. However, the minor differences observed may be a sampling artifact with these species sharing a common gene pool.

The allele-based method we have used helps alleviate the problem of excess discrimination in *Campylobacter* typing. Many alleles show differences in frequency between hosts. These alleles provide useful information on source for STs that are too rare to allow estimates of their frequency in different hosts (e.g., because they are absent from training sets).

Our approach has some limitations that must be considered in any more extensive application. The current accuracy estimate of 80% in distinguishing chicken isolates from bovid ones may be optimistic if sampling effects are quantitatively important. Sampling effects would include the nature of the sample (feces, meat), as well as time and place. For example, the dominant *Campylobacter* types found in processed carcasses have been shown to differ from those found in live chickens entering the processing plant (*19*). Nonetheless, we have shown that easily identifiable sampling effects are overwhelmed by the host

Table 5. Animal subpopulations used to study the effect of time and sample location on isolates of *Campylobacter jejuni*

Source*	No. animals
Early UK chickens	114
Late UK chickens	78
All UK chickens	217
Dutch chickens	53
Non-UK chickens	92
Late UK cattle and sheep	273

*Early, 1990–1997; late, 1998–2003.

effect. Moreover, because analysis within the ST-21 complex (Figure) is robust to identified and unidentified sampling effects, we do not believe this to be a major problem.

An additional limitation of our allele-based application of STRUCTURE is that it assumes allelic independence, which is clearly violated for the dataset analyzed. Assignment accuracy can be estimated in 2 different ways. The first, which we have used throughout this report, is a holdout procedure whereby source of origin of strains for which the actual origin is known is predicted by using the rest of the sample as a training set. This method provides an unbiased empiric measure of accuracy. To predict isolates for which the source is unknown, this procedure is not possible. Thus, it would be desirable to use estimates of accuracy that the algorithm provides. Because STRUCTURE assumes each allele is independent, its estimate of the accuracy with which it estimates the frequency of a particular multilocus genotype frequency is often overconfident. For example, in differentiating chicken isolates from those originating in cattle and sheep, STRUCTURE predicts an accuracy of 91%, but empirically it achieves an average accuracy of 80%. A better estimate of uncertainty would be necessary for predictive purposes. More sophisticated genetic models that reflect dependence among loci should achieve more accurate assignment as well as better estimates of statistical uncertainty.

Despite these limitations, this approach demonstrates the ability to assign isolates probabilistically to populations. When broad reference populations from the full range of possible sources are available, groups of isolates, such as those affecting a human population over a period of time, can be apportioned to their sources. However, precision in the attribution of *C. jejuni* may be less than that of *Salmonella*, in which different animal and bird species appear to host more differentiated populations (*3*). Prediction is possible with individual isolates, in some instances to 1 source, although prediction may suggest a range of populations rather than 1 population. For example, 2 of the sequenced *C. jejuni* genomes are from known sources, 1 from a chicken (isolate RM-1221) (*20*) and 1 from a human with campylobacteriosis who had drunk raw milk (isolate 81-176) (*17,18*). Assigning these isolates on the basis of reference datasets we used in this report predicted their origin as chicken (99% probability) and cattle/sheep (97% probability), respectively.

The broad host range of *C. jejuni*, spanning a variety of mammalian, avian, and other species, makes it a good model for studying features that may be informative of the ecology of multihost pathogens. *C. jejuni* acquires genome fragments estimated to be a few hundred bp in length (*21*) from other members of the species. Our analysis of the ST-21 complex shows that isolates in this complex have acquired genetic material prevalent in the population of *Campylobacter* carried by their host species (Figure). This observation implies that there is persistent differentiation in allele frequencies between different host species and that many ST-21 isolates represent lineages that have persisted within the same host species long enough to acquire a substantial number of alleles.

We surveyed 7 loci and found an average of 0.32 host-specific alleles in 81 STs other than ST-21 that were members of ST-21 complex, i.e., ≈5% of the alleles in this analysis. The acquired genes were approximately evenly distributed between these types, with 6 of the 7 loci having ≥1 import. The MLST loci were chosen because they represent core metabolic functions of *C. jejuni* (*10*) and are not obvious candidates for host adaptation. Therefore, we are likely observing the neutral level of genetic import. Extrapolating linearly from these 7 loci to 1,654 gene-coding sequences in the *C. jejuni* genome (*22*) gives an estimate of 76 genes with alleles typical of a particular host species within each ST-21 complex isolate. This estimate is rough because it is based on fairly limited data and recombination and selection at other genes may be different. However, this approximation shows the potential for substantial adaptation to the most recent host by homologous recombination. Homologous recombination may be an important factor in allowing a bacterial species to colonize a wide range of host species stably while adapting to some extent to each host.

In conclusion, a population genetic approach has allowed host assignment for *C. jejuni* for which host-specific markers are unavailable but host species populations are differentiated by allele frequency at a range of loci. Host association appears stronger than temporal and geographic effects. Homologous recombination generates a host signature in the *C. jejuni* genome and analyses that use this signal have improved accuracy of host prediction. The inherent standardization and portability of sequence typing in combination with the availability of such improved assignment techniques support the application of this approach to clarify aspects of *C. jejuni* epidemiology on a global scale and to study other suitable microbes.

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Dr McCarthy is a research fellow at Oxford University and honorary consultant epidemiologist at the Health Protection Agency. His research interests center on the application of scientific methodology to public health problems in infectious disease control.

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Address for correspondence: Noel D. McCarthy, Department of Zoology, University of Oxford, South Parks Rd, Oxford OX1 3SY, United Kingdom; email: noel.mccarthy@zoo.ox.ac.uk

Meningococcal Disease in South Africa, 1999–2002

Garry B. Coulson,* Anne von Gottberg,* Mignon du Plessis,* Anthony M. Smith,* Linda de Gouveia,* and Keith P. Klugman,*† for the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA)[1]

We describe the epidemiology of invasive meningococcal disease in South Africa from August 1999 through July 2002, as reported to a laboratory-based surveillance system. *Neisseria meningitidis* isolates were further characterized. In total, 854 cases of laboratory-confirmed disease were reported, with an annual incidence rate of 0.64/100,000 population. Incidence was highest in infants <1 year of age. Serogroup B caused 41% of cases; serogroup A, 23%; serogroup Y, 21%; serogroup C, 8%; and serogroup W135, 5%. Serogroup B was the predominant serogroup in Western Cape Province, and disease rates remained stable. Serogroup A was most prevalent in Gauteng Province and increased over the 3 years. On pulsed-field gel electrophoresis analysis, serogroup A strains showed clonality, and serogroup B demonstrated considerable diversity. Selected isolates of serogroup A belonged to sequence type (ST)-1 (subgroup I/II) complex, serogroup B to ST-32/electrophoretic type (ET)-5 complex, and serogroup W135 to ST-11/ET-37 complex.

Despite progress in our understanding of the epidemiology of meningococcal disease, infection with *Neisseria meningitidis* continues to be a serious public health concern worldwide. Although occurring predominantly as sporadic disease with seasonal variation in most parts of the world, the highest burden of meningococcal disease occurs in the "meningitis belt" of sub-Saharan Africa, where epidemics are observed regularly (*1*). Historically these epidemics were associated with serogroup A and, to a lesser extent, serogroup C. However, serogroup W135 has recently emerged as a cause of epidemic disease in Africa (*2*,*3*), after outbreaks in 2000 and 2001 in Saudi Arabia during the annual Hajj pilgrimage to Mecca (*4*–*6*). Epidemics of meningococcal disease have occurred in Africa outside the meningitis belt (*7*,*8*).

Meningococcal disease associated with epidemics in Africa is generally caused by a limited number of genetically defined clonal groups (*9*,*10*). The 3 serogroup A pandemic waves reaching the African meningitis belt were caused by clones of subgroup III (*11*,*12*), and the recent outbreaks of W135 in West Africa were caused by strains belonging to the ET-37 complex (*3*,*13*).

In South Africa, meningococcal disease (a clinically reportable condition since 1920) is endemic, with seasonal increases during the winter months (*14*,*15*). Incidence rates, as determined by clinical notifications to the Department of Health, have been steadily decreasing from ≈5–10/100,000 (1945–1975) to <2/100,000 (1992–1997) (*16*–*18*). Upsurges of disease with a periodicity of several years have been noted (*17*). During the late 1970s, the epidemiology changed from a preponderance of disease due to serogroup A in young adult black men on the gold mines in the Southern Transvaal (now Gauteng) Province, to mostly serogroup B disease affecting young mixed-race infants in Western Cape Province (*15*,*16*). Serogroup B has caused peaks in disease rates in Western Cape in 1979 (*9*,*19*–*21*) and again in 1988. Although predominant in Western Cape, serogroup B also caused cases in Johannesburg, Gauteng, during 1980–1982, where >60% of meningococcal disease in children was due to serogroup B (*22*). Increases in serogroup A disease in Gauteng were described in the 1980s and 1996 (*18*,*23*,*24*).

*National Institute for Communicable Diseases of the National Health Laboratory Service, Johannesburg, South Africa; and †Emory University, Atlanta, Georgia, USA

[1]GERMS-SA: Sandeep Vasaikar (Eastern Cape); Nolan Janse van Rensberg, Peter Smith (Free State); Khatija Ahmed, Heather Crewe-Brown, Mike Dove, Charles Feldman, Alan Karstaedt, Olga Perovic, Pyu-Pyu Sein, Ruth Lekalakala (Gauteng); Wim Sturm (KwaZulu Natal); Ken Hamese (Limpopo); Keith Bauer (Mpumalanga); Denise Roditi, Rena Hoffmann, Lynne Liebowitz, John Simpson, Andrew Whitelaw (Western Cape); Adrian Brink (AMPATH); Claire Heney (Lancet); Martinus Senekal (PathCare).

To better understand the recent epidemiology of invasive meningococcal disease in South Africa, we analyzed cases reported to a national laboratory-based surveillance system for a 3-year period, from August 1999 through July 2002. Isolates available from cases reported during this period were characterized further.

Materials and Methods

Case Definition

National laboratory-based surveillance for invasive disease caused by *N. meningitidis* is performed by the Respiratory and Meningeal Pathogens Research Unit (RMPRU) at the National Institute for Communicable Diseases (a branch of the National Health Laboratory Service) in Johannesburg, South Africa. Cases were defined as isolation of *N. meningitidis* from normally sterile body fluid specimens (blood, cerebrospinal fluid [CSF], or both) from patients in August 1999 through July 2002. Isolates were submitted voluntarily to RMPRU by ≈100 laboratories nationally. Laboratories were encouraged to submit case reports of laboratory-confirmed disease even if viable isolates were no longer available for submission. (Some isolates lost viability during transport to the central laboratory.) Annual audits were performed to ascertain missed cases, and these were included on the database (but were without viable isolates for further testing at the central laboratory). These audits identified 118 cases not reported, to reach a final total of 557 cases from the provinces/laboratories audited, which suggests that ≈70%–80% of laboratory-confirmed cases were reported to the surveillance system.

Serogrouping

Serogroup was determined for 615 isolates by using latex slide agglutination with monoclonal antiserum to capsular polysaccharides A, B, C, X, Y, Z, and W135 (Murex Biotech Limited, Dartford, England, United Kingdom). Strains that did not react with these antisera were sent to the World Health Organization Collaborating Center for Reference and Research on Meningococci, Oslo, Norway, for serogrouping.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed on 573 viable isolates of serogroup A, B, C, W135, and Y meningococci by using a method adapted from Popovic et al. (*25*). PFGE restriction profiles were analyzed with the GelCompar version 4.1 software (Applied Maths, Kortrijk, Belgium). Dendrograms were created by using the unweighted pair group method with arithmetic averages. Analysis of the banding patterns was performed with the Dice coefficient and a position tolerance of 1.5% for the band migration distance. A PFGE cluster was defined as >3 isolates sharing ≥80% similarity on the dendrogram (*25*,*26*).

Multi-Locus Sequence Typing (MLST)

MLST was performed on 46 isolates as described by Maiden et al. (*27*). We made use of the Neisseria MLST website (http://pubmlst.org/neisseria/) sited at the University of Oxford (*28*).

Statistical Analysis

Incidence rates were calculated on the basis of the number of cases reported during the 12-month periods from August 1 through July 31 of the following year, divided by mid-year population estimates for years 2000, 2001, and 2002, respectively, obtained from the South African Health Information Systems Programme. The χ^2 test for linear trend using EpiInfo 6 (version 6.04d; Centers for Disease Control and Prevention, Atlanta, Georgia, USA) was used to assess statistical significance of the changes during the 3-year period.

Results

Epidemiology of Laboratory-confirmed Meningococcal Disease

From August 1999 through July 2002, 854 cases of invasive meningococcal disease were reported; age was known for 756 (88%) patients. Most cases (645, 76%) were diagnosed from positive culture of CSF specimens (with or without positive cultures from blood specimens); the other 209 (24%) were positive on blood culture alone. The incidence rates of disease reported to the network increased from 0.52 per 100,000 persons in 1999–2000, to 0.62 in 2000–2001, and 0.77 in 2001–2002 ($p<0.001$). Western Cape Province was responsible for 37% of cases reported nationally, and Gauteng Province was responsible for 41% of cases reported nationally (Figure 1). In Western Cape Province, disease rates remained relatively stable; rates of reported disease were calculated as 2.87/100,000, 1.91/100,000, and 2.27/100,000 for each 12-month period, respectively ($p = 0.068$) (Figure 2). The incidence rates in Gauteng Province increased from 0.54/100,000 in the first year to 1.42/100,000 and 1.99/100,000 in the subsequent 2 years ($p<0.001$) (Figure 3). Seasonal variation was observed; the highest number of cases was reported in July to October (winter and spring) (data not shown). The highest age-specific incidence of meningococcal disease was seen in infants <1 year of age; the average incidence rate was 6.7/100,000. One hundred eighty-two (24%) of patients were infants <1 year of age, 116 (15%) were children 2–4 years of age, and 127 (17%) were young adults 15–24 years of age.

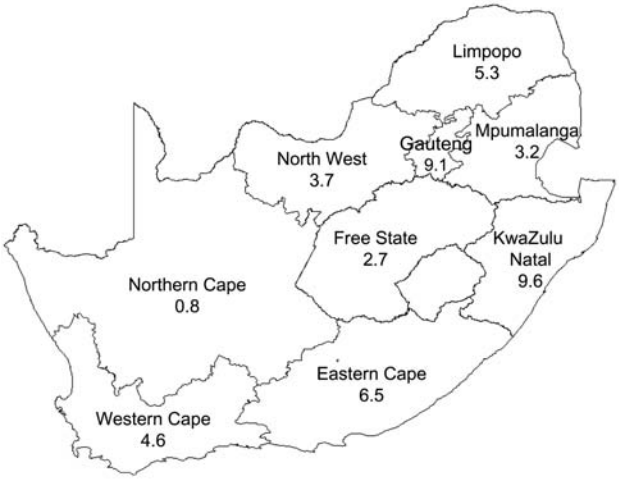

Figure 1. Map of South Africa with estimated provincial populations in 2002 (45.5 million population). Values are in millions.

Serogroup-specific Disease

Of the 854 cases of laboratory-confirmed meningococcal disease reported to the surveillance network, 615 (72%) had viable isolates available for serogrouping; 453 (74%) of these were isolated from CSF and 162 (26%) from blood culture alone. Serogrouping of the viable meningococcal isolates showed the following: serogroup B, 251 isolates (41%); A, 142 (23%); Y, 130 (21%); C, 50 (8%); W135, 31 (5%); X, 8 (1%); Z, 2 (<1%); and 29E, 1 (<1%) (Table 1).

Seventy percent (175/251) of serogroup B disease was reported from Western Cape Province, where the number of cases decreased progressively from 81 in the first year (1999–2000) to 38 (in the third year, 2001–2002) (Table 1) and incidence decreased from 1.85/100,000 to 0.82, respectively ($p<0.001$) (Figure 2). Eighty-five percent (121/142) of serogroup A disease came from Gauteng Province, and the annual number of cases increased from 9 to 78 during the study period (Table 1). The incidence rate increased from 0.11/100,000 in the first year to 0.86 in the third year ($p<0.001$) (Figure 3). The proportion of disease caused by serogroups C, W135, and Y remained stable during the 3-year period (Table 1). Serogroup W135 was most prevalent in Gauteng Province (19 [61%] of the 31 cases occurred there), and serogroup C was most prevalent in Western Cape Province (28 [56%] of 50).

The age-specific proportion of disease in patients with known age varied for serogroups. The highest proportion of serogroup A (38 [33%] of 114) and C (10 [20%] of 50) disease occurred in the 15- to 24-year age group; the highest proportion of disease caused by serogroup B (70 [29%] of 238) and Y (42 [38%] of 112) was in infants <1 year of age. Serogroup W135 was found in equal proportion in the <1-year age group (6 [23%] of 26) and 15–24 age group (7 [27%] of 26). Incidence rates for the most common serogroups (A, B, and Y) for the last year of surveillance showed the highest rates of disease in children <1 year of age (Figure 4). Serogroup A had the lowest rates of disease for infants of the 3 serogroups and also had a second small peak for young adults. These trends were similar in the previous 2 years.

Of 615 *N. meningitidis* isolates serogrouped, 573 (93%) isolates were characterized by PFGE. Forty-six of these isolates were selected for MLST (Table 2).

Molecular Epidemiology

Serogroup A

PFGE analysis of 123 serogroup A isolates showed a highly clonal population structure with a large cluster (cluster A-1) representing 89% (109/123) (Figure 5, Table 2). The proportion of serogroup A meningococcal disease associated with strains of cluster A-1 increased from 38% (5/13) in 1999–2000 to 97% (70/72) in 2001–2002 ($p<0.001$). Most isolates from this cluster (101/109, 93%) originated from Gauteng Province and increased from 56% (5/9) in the first year, to 97% (30/31 and 66/68, respectively) in the second and third years ($p<0.001$). MLST analysis of 12 isolates from cluster A-1 showed identical allelic profiles belonging to sequence type (ST)-1, the prototype ST for the ST-1 (subgroup I/II) complex (Table 2). MLST analysis of 4 isolates outside of cluster A-1 yielded strains belonging to ST-7 (n = 2), ST-254 (n = 1), and ST-175 (n = 1) (Table 2).

Serogroup B

In total, 242 serogroup B *N. meningitidis* isolates were analyzed by PFGE. Five distinct clusters were observed, with a predominant cluster (cluster B-1) consisting of 38% (91/242) of the isolates (Figure 6). The proportion of isolates within this cluster was 36%, 36%, and 43% for each 12-month period, respectively ($p = 0.369$). Eighty-two

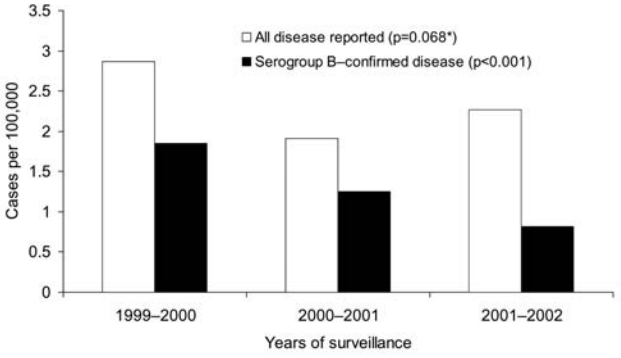

Figure 2. Incidence rates for all reported and serogroup B–confirmed meningococcal disease by year in Western Cape Province. *χ^2 test for trend.

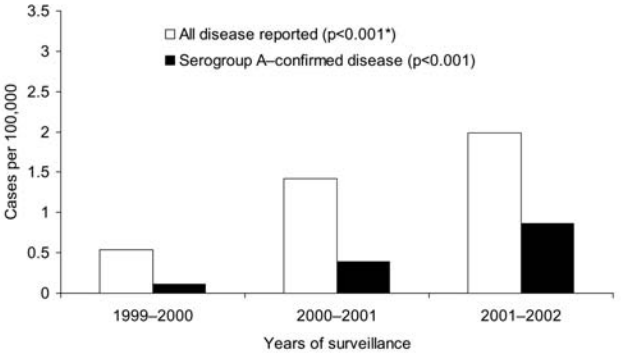

Figure 3. Incidence rates for all reported and serogroup A–confirmed meningococcal disease by year in Gauteng Province. *χ^2 test for trend.

percent (75/91) of the isolates from this cluster were from the Western Cape, and the proportion of these strains in this province remained stable over time. Five isolates from this cluster were selected for MLST analysis. Four isolates were ST-33 (Table 2). One isolate had a novel allele at the *fumC* locus (*28*); a new ST (ST-4239, still part of ST-32 complex) was assigned to this isolate.

The second largest cluster (cluster B-2) comprised 12% (29/242) of the total number of isolates characterized. Three isolates belonged to ST-41/44 lineage III, 2 of which were ST-154. The third isolate had a novel allele at the *abcZ* locus and was assigned ST-4242.

Clusters B-3, B-4, and B-5 comprised 9.5% (23/242), 7% (17), and 6% (14) of all serogroup B isolates, respectively. The remaining isolates were clustered into small groups or were unrelated.

Serogroup C

PFGE of the 49 serogroup C meningococcal isolates showed 3 main clusters (clusters C-1, C-2, and C-3) (Table 2). Cluster C-1 comprised 31% (15/49) of the total number of serogroup C isolates and showed no particular concentration by province. Two isolates from this cluster were ST-11 (Table 2).

Isolates belonging to clusters C-2 and C-3 each made up 18% (9/49) of the total number of isolates characterized (Table 2). Isolates from both clusters came exclusively from Western Cape (18/18 isolates). MLST of 2 strains from each cluster identified ST-865 in cluster C-2 (an ST not associated with any broader ST complex), and ST-33 in

Table 1. Provincial distribution of reported invasive meningococcal disease by serogroup and date of study, South Africa

Year	Serogroup	Province*									Total, n (%)†
		EC	FS	GA	KZ	LIM	MP	NC	NW	WC	
Aug 1999–Jul 2000	A	–‡	–	9	1	–	–	–	1	4	15 (8)
	B	6	–	12	4	–	3	–	–	81	106 (56)
	C	2	2	2	–	–	–	–	–	14	20 (10)
	W135	–	–	3	3	–	–	–	–	4	10 (5)
	X	–	–	1	–	–	–	–	–	2	3 (2)
	Y	2	6	10	7	–	–	–	–	11	36 (19)
	No isolate available	2		9	10	4	2		2	10	39
	Total	12	8	46	25	4	5	–	3	126	229
Aug 2000–Jul 2001	A	–	–	34	2	–	5	–	3	–	44 (23)
	B	6	3	13	3	–	1	–	1	56	83 (43)
	C	3	–	3	–	–	1	–	1	4	12 (6)
	W135	–	–	7	–	–	–	–	–	1	8 (4)
	X	–	–	1	–	–	–	–	–	1	2 (1)
	Y	2	14	13	1	2	3	1	1	5	42 (22)
	Z	–	–	2	–	–	–	–	–	–	2 (1)
	No isolate available	3		51	2	6	1			19	82
	Total	14	17	124	8	8	11	1	6	86	275
Aug 2001–Jul 2002	A	–	–	78	1	–	1	–	2	1	83 (36)
	B	3	4	15	–	–	–	1	1	38	62 (27)
	C	1	–	6	–	–	–	1	–	10	18 (8)
	29E	–	–	–	–	–	–	–	–	1	1 (<1)
	W135	1	–	9	–	–	–	–	1	2	13 (6)
	X	–	–	2	–	–	–	–	–	1	3 (1)
	Y	3	14	20	4	–	1	2	1	7	52 (22)
	No isolate available	3	2	51	5		10	1	1	45	118
	Total	11	20	181	10	–	12	5	6	105	350

*EC, Eastern Cape; FS, Free State; GA, Gauteng; KZ, KwaZulu Natal; LIM, Limpopo; MP, Mpumalanga; NC, Northern Cape; NW, North West; WC, Western Cape.
†Percentages denote the percentage of that particular serogroup over the total number of serogrouped isolates for that year.
‡No cases reported.

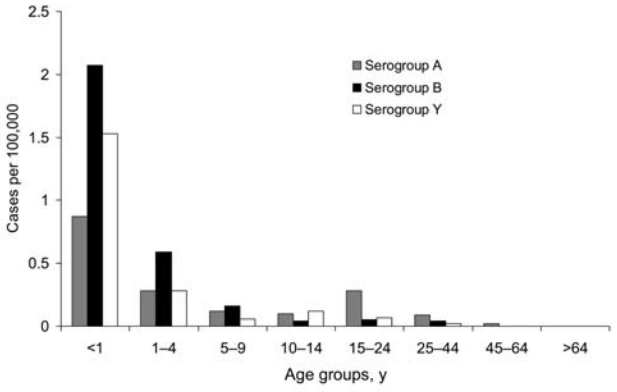

Figure 4. Annual age-specific incidence rates for confirmed serogroup A, B, and Y meningococcal disease in South Africa, as reported from August 2001 through July 2002.

cluster C-3 (Table 2). The remaining isolates all showed unrelated PFGE patterns.

Serogroup W135

Of the 31 serogroup W135 meningococci isolates analyzed by PFGE, a distinct cluster (cluster W-1) of isolates comprising 23 (74%) of 31 isolates was found (Table 2). Seventeen (74%) of cluster W-1 isolates came from Gauteng Province. MLST analysis of 4 isolates from cluster W-1 (2 isolates from Gauteng and 1 each from Western Cape and KwaZulu Natal Provinces) showed they were ST-11, the founder sequence type of the ST-11/electrophoretic type (ET)-37 complex. Cluster W-2 comprised 3 isolates from 3 provinces, and MLST of 1 of the isolates showed that it belonged to ST-4241 (ST-22 complex). The remaining isolates were unrelated.

Serogroup Y

PFGE analysis of the 128 serogroup Y meningococcal isolates showed 2 clusters (clusters Y-1 and Y-2; Table 2). The predominant cluster (cluster Y-1) consisted of 92 (72%) isolates. Twenty-five (27%) isolates from cluster Y-1 came from Free State Province, 27 (29%) from Gauteng Province, and 15 (16%) from Western Cape Province. MLST of 6 isolates from this cluster showed that they were ST-175 (Table 2).

A second cluster, cluster Y-2, comprised 15% (19/128) of isolates (Table 2). Fifty-three percent (10/19) of these isolates were from Gauteng Province. MLST analysis of 3 isolates showed 2 STs, 1 identified as ST-23 (2 isolates), with the third possessing a novel allele at the *abcZ* locus (assigned new ST-4245). The remaining isolates (17/128, 13%) demonstrated groups of 2, 3, or 5 isolates; and 4 unrelated isolates. MLST analysis of 1 of these isolates showed that it belonged to ST-175.

Discussion

The endemic nature and low incidence rates of meningococcal disease in the study period confirm an epidemiology related more closely to industrialized countries (*29*,*30*) than to countries of the African meningitis belt. Rates of national disease, as calculated by clinical notifications, ranged between 1 and 2/100,000 from 1992 to 1997 (*18*) and are similar to those calculated in our study. Although laboratory-based surveillance in South Africa clearly underestimates the impact of disease, audits indicate that more than two thirds of laboratory-confirmed disease were reported, and we believe our data are representative enough to reflect general trends of disease.

Overall, the age group at greatest risk for disease was children <1 year of age, although there were some differences by serogroup. Serogroup B has been previously described to occur predominantly in infants (*15*,*22*,*30*); serogroup A disease also causes disease in adults (*15*,*22*,*24*). Serogroup Y disease occurring in older patients has been documented (*30*), but this was not observed in our study.

The high proportion of laboratory-confirmed cases from Gauteng and Western Cape Provinces could reflect better reporting by laboratories in these areas. These 2 provinces also had the most clinical notifications, which would be less reliant on laboratory facilities, to the Department of Health since the 1970s (*18*). Other parts of South Africa were noted to have much lower rates of disease (*15*). Although access to medical care may influence rates by province, the fulminant and distinctive clinical manifestations of meningococcal disease allow for adequate clinical reporting from health facilities. True environmental, socioeconomic, or host-related factors may be resulting in higher disease rates in these provinces. Climate varies between areas in the country: Western Cape has a Mediterranean climate with wet winters and hot, dry summers; Gauteng lies on a plateau and has a temperate climate with summer rainfall; and KwaZulu Natal has a predominantly subtropical climate (*31*).

The incidence rate of reported meningococcal disease increased from 1999 to 2002, and serogroup A, most prevalent in Gauteng Province, was the only serogroup of viable isolates to increase significantly. Cyclical changes in meningococcal disease occurring every 8 to 10 years have been noted in this province (*18*). Case ascertainment of prospectively reported cases may have increased as the surveillance became more established and as audits highlighted nonreporting from certain laboratories that were subsequently included in the surveillance. Serogroup A meningococci are associated with most outbreaks throughout the African meningitis belt (*1*). No discrete outbreaks were identified associated with serogroup A disease during

Table 2. Genotypic data of *Neisseria meningitidis* isolates causing invasive disease as reported in South Africa, August 1999–July 2002*

Serogroup	PFGE clusters	No. of isolates				MLST results		
		1999/2000, n	2000/2001, n	2001/2002, n	Total, n (%)	ST	ST complex	n
A	Total	13	38	72	123			
	Cluster A-1	5	34	70	109 (89)	1	ST-1/subgroup I/II	12
	Small clusters/ single isolates	8	4	2	14 (11)	7	ST-5/subgroup III complex	2
						254	ST-254 complex	1
						175	None	1
B	Total	107	77	58	242			
	Cluster B-1	38	28	25	91 (38)	33	ST-32/ET-5 complex	4
						4239	ST-32/ET-5 complex	1
	Cluster B-2	12	7	10	29 (12)	154	ST-41/44/lineage III	2
						4242	ST-41/44/lineage III	1
	Cluster B-3	11	9	3	23 (9.5)			
	Cluster B-4	7	4	6	17 (7)	35	ST-35 complex	1
	Cluster B-5	3	6	5	14 (6)			
	Small clusters/ single isolates	36	23	9	68 (28)			
C	Total	20	12	17	49			
	Cluster C-1	5	5	5	15 (31)	11	ST-11/ET-37 complex	2
	Cluster C-2	3	2	4	9 (18)	865	None	2
	Cluster C-3	4	1	4	9 (18)	33	ST-32/ET-5 complex	2
	Small clusters/ single isolates	8	4	4	16 (33)			
W135	Total	9	9	13	31			
	Cluster W-1	5	7	10	23 (74)	11	ST-11/ET-37 complex	4
	Cluster W-2	2	1	0	3 (10)	4241	ST-4241/ST-22 complex	1
	Small clusters/ single isolates	2	1	3	5 (16)			
Y	Total	40	38	50	128			
	Cluster Y-1	28	25	39	92 (72)	175	None	6
	Cluster Y-2	5	8	6	19 (15)	23	ST-23 complex/ cluster A3	2
						4245	ST-23 complex/ cluster A3	1
	Small clusters/ single isolates	7	5	5	17 (13)	175	None	1

*PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type.

the study period; however, unrecognized clusters may have occurred.

The increase in the number of cases of serogroup A reported from Gauteng Province was associated specifically with strains belonging to a distinct cluster identified by PFGE. Selected isolates from this cluster were confirmed as belonging to ST-1 (subgroup I/II) complex. These strains have caused epidemics worldwide (*11*,*32*,*33*). In South Africa, subgroup I strains were first identified in 1968 (1 isolate) (*11*,*32*) and from 1976 through 1983 (41 isolates) (*32*). In 1996, 49.5% (55/111) of isolates analyzed from an outbreak in South Africa were identified as serogroup A belonging to subgroup I; 13.5% (15/111) belonged to subgroup III (*34*). MLST analysis of 1 of these subgroup III isolates showed it was ST-5 (data not shown). Recently, in the meningitis belt, ST-5 (predominant in 1988–2001) has been replaced by ST-7 in 2002, and no ST-1 strains were identified (*35*). In our study, 2 isolates analyzed by MLST were confirmed as ST-7, which suggests that the third pandemic wave from People's Republic of China may have reached South Africa (*12*,*35*). The predominant serogroup A strain causing disease in South Africa, however, was not the same strain as that in the meningitis belt.

The high proportion of sporadic serogroup B disease in the Western Cape has been well described since the late 1970s (*15*,*19*,*36*). Serogroup B is rarely reported from other countries in Africa, and our data reflect an epidemiology for this serogroup more consistent with industrialized countries (*9*,*37*). The proportion of serogroup B meningococcal disease nationally decreased significantly, mostly due to a decrease in the number of viable serogroup B isolates identified from the Western Cape. This province had no change in total reported disease rates. By PFGE this

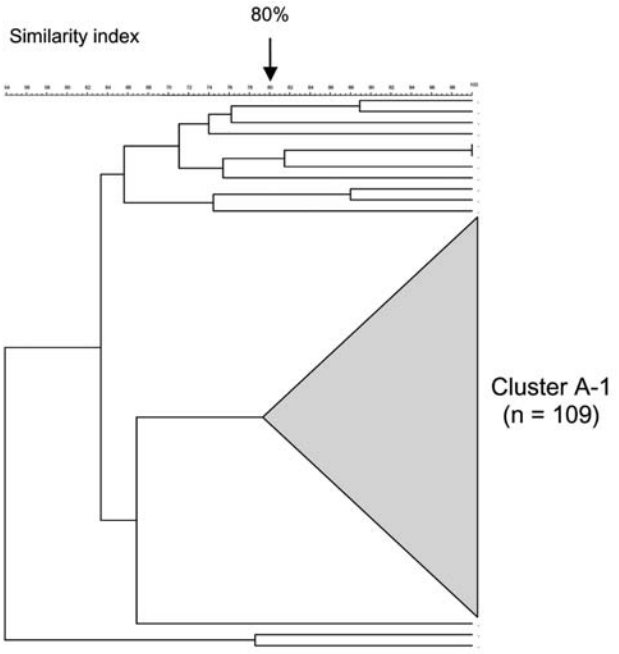

Figure 5. Pulsed-field gel electrophoresis dendrogram indicating the genetic relationship among serogroup A meningococcal isolates in South Africa, August 1999–July 2002.

serogroup showed substantial diversity, a characteristic typical of sporadic serogroup B disease worldwide (*30*,*38*) and previously documented in the Western Cape (*39*). Complexes ST-32/ET-5 and ST-41/44/lineage III have been associated with outbreaks worldwide (*9*,*37*). These strains have been causing disease in the Western Cape since the late 1970s (*9*,*21*,*39*).

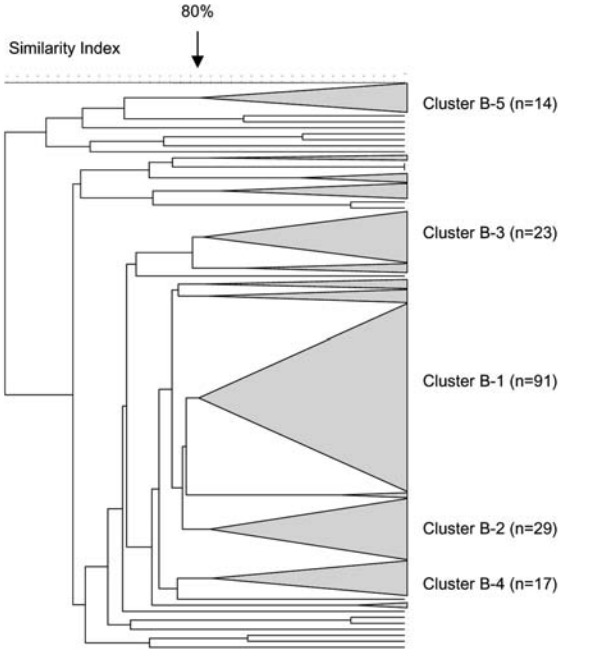

Figure 6. Pulsed-field gel electrophoresis dendrogram indicating the genetic relationship among serogroup B meningococcal isolates in South Africa, August 1999–July 2002.

Serogroup Y accounts for approximately one third of all invasive meningococcal disease in the United States (*30*), but it has been rare in the African meningitis belt (*1*,*35*). A serogroup Y isolate with ST-175 has been previously described from The Gambia in 1988 (www.pubmlst.org/neisseria), and recently ST-23 and ST-2880 have been identified in the meningitis belt (*35*). Our data thus represent the first evidence of a major role for serogroup Y disease in Africa.

Serogroup C disease associated with sporadic disease and occasional outbreaks occurs in both industrialized and developing nations (*1*,*30*). Complexes ST-11/ET-37 and ST-32/ET-5 are hypervirulent meningococci reported worldwide (*9*,*37*). In South Africa, a community-based outbreak caused by strains of the ST-11/ET-37 complex was observed in 2003 (*40*). ST-865 strains have been reported to cause disease in Taiwan, Spain, and the United States (www.pubmlst.org/neisseria), but these were associated with nongroupable and non–serogroup C isolates. To our knowledge, we document the first serogroup C strain of ST-865.

Serogroup W135, associated with little disease worldwide (*1*,*13*), represented a small proportion of disease in our surveillance. Complex ST-11/ET-37 was responsible for outbreaks in 2000 and 2001 associated with the annual Hajj pilgrimage (*4*,*6*,*13*) and for outbreaks in Burkina Faso in 2001 (*2*). PFGE comparison of isolates from a predominant cluster in South Africa with an isolate from the Hajj outbreak showed that they were related (data not shown). Strains of ET-37 had been in South Africa in 1986 and 1990 (*13*), and this clone may have been reintroduced during the Hajj outbreak.

In conclusion, we identified sporadic and seasonal meningococcal disease in South Africa during the study period, caused in part by an increasing number of cases due to a clone of serogroup A in Gauteng Province. Diverse strains of serogroup B were responsible for stable prevalence of disease in Western Cape Province. Nationally, 21% of meningococcal disease was due to serogroup Y. Continued surveillance will provide valuable information for the development of public health strategies to minimize the risk for outbreaks in South Africa and neighboring countries.

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Mr Coulson worked for the National Institute for Communicable Diseases, Johannesburg, South Africa, at the time this article was prepared. Currently, he is in the doctoral program in infectious diseases at the University of Georgia, Athens, USA. His research activities focus on the role of genes on the pathogenicity island in *Rhodococcus equi* in terms of survival and persistence in host macrophages.

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Address for correspondence: Anne von Gottberg, Respiratory and Meningeal Pathogens Research Unit, National Institute for Communicable Diseases, Private Bag X4, Sandringham, 2131, Gauteng, South Africa; email: annev@nicd.ac.za

Neutralizing Antibodies after Infection with Dengue 1 Virus

Maria G. Guzman,* Mayling Alvarez,* Rosmari Rodriguez-Roche,* Lídice Bernardo,* Tibaire Montes,† Susana Vazquez,* Luis Morier,* Angel Alvarez,* Ernest A. Gould,‡ Gustavo Kourí,* and Scott B. Halstead§

Severity of disease is markedly increased when infection with dengue virus type 2 (DENV-2) follows infection with DENV-1 at an interval of 20 years. Studies have shown that heterologous neutralizing antibody titers are inversely correlated with severity of a second infection. If this mechanism controlled disease severity in Cuba, heterotypic antibody titers should have declined over time. To determine whether phenotypic changes in dengue antibodies occur over time, we analyzed serum samples collected 4–8 and 20–22 years after DENV-1 infection. We found a significant increase in mean titer of homologous DENV-1 neutralizing antibodies and a significant decrease in heterologous antibodies to 1 of 2 genotypes of DENV-2 virus (the American genotype). Asian DENV-2 viruses were not neutralized during either interval; however, the American genotype underwent phenotypic changes in heterotypic viral neutralizing antibodies in the predicted direction. This finding may be related to the time-dependent changes in severity of disease found with secondary dengue infection.

During 1977, Cuba experienced a nationwide outbreak of dengue fever (DF). More than 500,000 cases caused by a dengue 1 virus (DENV-1) of Southeast Asian origin were reported (*1*,*2*). Seroepidemiologic studies during 1978–1979 demonstrated hemagglutination-inhibition antibodies against dengue virus in 44.46% of the population (*1*). In 1981, an Asian genotype dengue 2 (DENV-2) produced a major islandwide epidemic of DF and dengue hemorrhagic fever (DHF); >400,000 cases were reported, 10,000 of which resulted in DHF and 158 deaths (101 children) from DHF (*3*–*8*). During 1982–1996, strong vector-control programs stopped transmission of dengue viruses.

In 1997, an Asian genotype DENV-2, closely related to the 1981 strain, entered Cuba and circulated in the island's second largest city, Santiago de Cuba, producing a severe outbreak of DF and DHF (*9*,*10*). At the time of the outbreak, 25%–35% of the population 18–54 years of age were monotypically immune to DENV-1 because of infections during 1977–1979 (*1*,*2*,*11*). During the 1997 epidemic, an estimated 4,810 adults experienced a second dengue infection with DENV-2, 18–20 years after infection with DENV-1 (*10*). Of this group, 205 patients were hospitalized with DHF, 12 of whom died. No cases of DHF or dengue shock syndrome were observed in children (*12*). When standardized for age, case-fatality rates for persons who had been infected with DENV-1 during 1977–1979 and secondarily infected with DENV-2 in 1997 were 3–4× higher than for persons who had secondary DENV-2 infections in 1981 (*13*). In addition, virtually all (≈100%) secondary DENV-2 infections in the 1997 Santiago de Cuba outbreak were clinically overt in marked contrast to primary DENV-2 infections, of which only 3.0% produced overt disease (*10*).

Both DENV-2 viruses, from 1981 and 1997, belong to the same genotype. Although amino acid differences in structural and nonstructural regions of the 2 genomes might contribute to the observed differences in disease severity, the low intrinsic virulence of the 1997 DENV-2 virus was remarkable. In this study, we focused on the possibility that presence or absence of heterotypic neutralizing antibodies might contribute to severity of secondary DENV-2 infections.

Several observations suggest this to be a mechanism for controlling dengue disease severity during heterotypic infections. The first such observations were made by

*"Pedro Kourí" Tropical Medicine Institute, Havana, Cuba; †Instituto Medicina Tropical, Caracas, Venezuela; ‡Centre for Ecology and Hydrology, Oxford, United Kingdom; and §Pediatric Dengue Vaccine Initiative, Bethesda, Maryland, USA

Sabin, who observed a 3-month period of cross-protection to DENV-2 illnesses after DENV-1 infections in human volunteers (*14*). When DENV-1–immune volunteers were challenged with DENV-2 at intervals >3 months, classical DF occurred. A comparable observation was made in a school-based study in Thailand (*15*), in which 40 children experienced predominantly secondary DENV-2 infections; of these, 33 were fully protected from disease accompanying DENV-2 infections and only 7 were hospitalized. Of the former, human monocyte assay of undiluted serum showed that 31 had DENV-2 neutralizing antibodies from a prior heterotypic dengue infection. By contrast, serum from 6 children who had severe disease did not have neutralizing antibodies, but it enhanced DENV-2 infections (*15*). A similar observation was made in Iquitos, Peru, where DENV-1 had been endemic since 1990. In 1995, an American genotype DENV-2 was introduced into this population that was already highly immune to DENV-1 (*16*). Despite large numbers of persons who were infected initially with DENV-1 and subsequently with DENV-2, no DHF cases were observed. Plaque-reduction neutralization test (PRNT) of DENV-1–immune human serum samples obtained in 1994 in Iquitos showed that nearly all contained high levels of neutralizing antibodies to American genotype but not Asian genotype DENV-2 viruses. The latter viruses have circulated for a long time in populations who are immune to multiple dengue viruses and who could plausibly have lost DENV-1–like epitopes by preferential selection of antibody escape mutants (*17*).

Thus, from these 2 studies we deduced that cross-reactive, dengue-neutralizing antibodies may down-regulate secondary dengue infections and prevent enhanced infections while mediating disease in persons with a different immune status. These observations are supported by recent studies in which DENV-1–immune monkeys were challenged with either American or Asian DENV-2 (*18*). On the basis of these observations, we examined whether dengue antibodies undergo phenotypic changes after many years; such changes would help explain the observed increase in disease severity accompanying secondary DENV-2 infections.

Materials and Methods

Serum Samples

Serum samples were submitted to our laboratory from a nationwide dengue surveillance program implemented during 1981–1985 (103 samples) and 1999 (2,000 samples). ELISA results showed no evidence of acute dengue infection. To avoid analyzing serum from persons infected by any other DENV, we excluded samples from Santiago de Cuba province because of the DENV-2 epidemic that occurred in 1997 (*19*,*20*).

Serum samples were first tested for dengue immunoglobulin G (IgG) by an ELISA inhibition method that used a DENV-1 antigen shown to provide the same or better sensitivity and specificity as tests that use all 4 dengue viruses. Samples with dengue IgG were retested by PRNT, which used strains of the 4 dengue serotypes, including 2 DENV-2 strains classified as either Asian (*3*,*4*,*8*) or American genotype (*21*) (Table 1). Testing for all dengue viruses by PRNT was conducted on BHK-21, clone 15 cells (*22*,*23*). Serum was diluted to 1:10, and then serial 10-fold dilutions were made in Earle's minimal essential medium (MEM). To obtain 15–20 plaques in a 24-well tissue culture plate, we mixed 100 μL of each serum dilution with 100 μL of media containing 80 PFUs of the assayed viruses and incubated this mixture at 37°C for 1 h. Then 50 μL of virus-serum mixture was added in triplicate onto 0.5 mL media containing 2.5×10^5 cells. After incubating this mixture for 4 h at 37°C in an atmosphere of 4.5% CO_2, we added 0.5 mL of overlay medium that contained 3% medium viscosity carboxymethylcellulose prepared in MEM without phenol red with 10% heat-inactivated fetal bovine serum, 1% glutamine (2 mmol/L), 100 U penicillin, and 100 μg/mL streptomycin. Infected cells were incubated for 5–9 d, depending on the virus serotype (7–9 d for DENV-1 and DENV-3, 5 d for DENV-2, and 6 d for DENV-4), under the same conditions. After incubation, plates were stained with a solution of naphthol blue-black dye and acetic acid, and the plaques were counted. Serum samples were tested simultaneously against each DENV strain; each serum dilution was tested in triplicate.

Antibody titers were expressed as the reciprocal of the endpoint dilution. For statistical purposes, samples with a titer <10 were assigned a titer of 5. Calculations of 50% endpoint plaque-reduction neutralization titers ($PRNT_{50}$) were made by using log probit paper and the method of Russell et al. (*24*). According to criteria previously established (*25*), samples with neutralizing antibody titers ≥30 to only 1 dengue virus were considered evidence of primary dengue infection. Considering the epidemiology of dengue in Cuba and using the DENV-2 strain that circulated during the 1981 epidemic, we classified samples that had dengue neutralizing antibodies ≥30 to DENV-1 but <5 for DENV-2 (A15/81 strain), DENV-3, and DENV-4 as a past primary DENV-1 infection during the 1977–1979 epidemic.

Statistical Analysis

For data analysis, we used GraphPad Prim 2.0 (SPSS Inc., Chicago, IL, USA). Neutralizing antibody titers were expressed as mean titers. Mean titers were compared to detect significant differences between antibody titers to viruses in each studied group and in both groups of samples by using 1-way analysis of variance followed by

Table 1. Dengue virus strains used in this study

Serotype	Strain	Passage no.*	Place and year of isolation
DENV-1†	Angola	4PC6/36 1PVero 1PC6/36	Angola, 1988
DENV-2‡	A15/81	4PMB 4PC6/36	Cuba, 1981
DENV-2†‡	I348600	4P C6/36	Colombia, 1986
DENV-3	116/00	3P C6/36	Cuba, 2000
DENV-4†	Dominica	7P C6/36	Dominica, 1981

*P, passage; C6/36, *Aedes albopictus* cell line; Vero, green monkey kidney cell line; MB, mouse brain.
†DENV, dengue virus.
‡DENV-2, A15/81 strain (Asian genotype), DENV-2, I348600 (American genotype).

the Bonferroni multiple comparison test. Statistical significance was defined as $p<0.05$. The Fisher exact test was used to compare the positive percentages of neutralizing antibody to each virus in each group of samples.

Results

Of the 103 serum samples collected during 1981–1985 and the 2,000 collected in 1999, dengue IgG antibodies were detected by screening ELISA inhibition method in 50 (48.5%) and 826 (41.3%), respectively. From these, the 50 samples in the first group (group 1) and 89 representative samples from the second (group 2) were classified as monotypic DENV-1–immune serum on the basis of $PRNT_{50}$ results with the 4 dengue serotypes.

Table 2 shows that the geometric mean titer of homologous neutralizing antibodies increased significantly in samples collected after 22 years compared with those collected 4–8 years after the DENV-1 epidemic of 1977. In contrast, over this same period, heterotypic antibodies directed against the American genotype of DENV-2 declined significantly in the number of samples that had heterotypic neutralizing antibodies to this genotype and in geometric mean titer. DENV-1–immune serum obtained years after inapparent infection showed little heterotypic neutralization of Asian DENV 2 (12%), DENV-3 (8%), or DENV-4 (2%) viruses.

The Figure shows each data point, together with mean $\log_{10}$ neutralizing antibody titers to the viruses tested in the studied groups. Means of DENV-1 antibodies differed significantly between groups 1 and 2. Means of antibody titers were significantly different ($p<0.001$) when DENV-2 (I/348600) was compared with DENV-3 and DENV-4 viruses. Significant differences ($p<0.001$) were also noted in heterotypic neutralization of DENV-2 (I/348600) in samples from groups 1 and 2. Means of DENV-2 (A 15/81), DENV-3, and DENV-4 did not differ between groups 1 and 2.

Discussion

We present 2 new findings. After DENV-1 infection, homotypic neutralizing antibody titers increase, and heterotypic antibody titers to 1 of 2 genotypes of DENV-2 virus (the American genotype) decrease.

However, our study had several limitations. One problem was that limited serum quantities precluded our ability to test for neutralization and enhancement in primary cultures of human monocytes. Another problem was that the effect of heterotypic neutralizing antibodies on the severity of DENV-2 infections during the 1981 epidemic should have been studied in a representative selection of samples collected before the 1981 outbreak from persons infected by DENV-1 during 1977–1979. Long-term kinetics of neutralizing antibodies requires that samples be collected at intervals from the same persons. To compensate for our inability to conduct longitudinal studies with the same persons, we studied relatively large numbers of samples from randomly selected persons who were immune to DENV-1. Serum available for study was sent to our diagnostic laboratory over a period of many years and stored at –20°C. Samples were sent from representative age and ethnic groups from all geographic areas of Cuba, excluding Santiago de Cuba province. We provide independent confirmation of the cross-neutralization of American genotype DENV-2 by antibodies raised to DENV-1 infections in Cuba.

Table 2. Neutralization of dengue viruses by dengue virus 1–immune serum collected 4–8 years (group 1) and 20–22 years (group 2) after primary infection*

	Group 1		Group 2	
Serotype (strain)	Positivity (%)	GMT	Positivity (%)	GMT
DENV-1 (Jamaica/77)	50 (100)	93	89 (100)	140.6
DENV-2 (A15/81)	6 (12)	5.5	19 (21)	6.5
DENV-2 (I348600)	36 (72)†	30	40 (45)‡	10.2
DENV-3 (116/00)	4 (8)	5.6	9 (10)	5.9
DENV-4 (Dominica)	1 (2)	5.1	13 (15)	6.2

*GMT, geometric mean titer; DENV, dengue virus.
†$p<0.00001$ compared with DENV-2 (A15/81), DENV-3, and DENV-4 viruses in group 1.
‡$p<0.01$–0.0001 compared with DENV-2 (A15/81), DENV-3, and DENV-4 viruses in group 2.

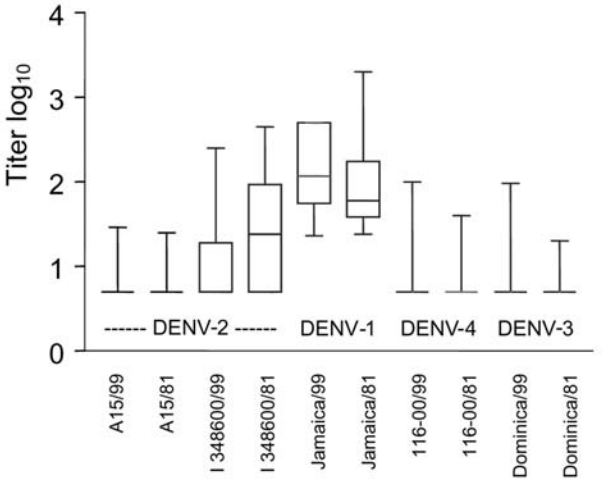

Figure. Log_{10} antibody titers for human dengue virus type 1 (DENV-1)–immune serum samples collected in 1999 (89 samples) and 1981–1985 (50 samples, mean).

Consistent with our research hypothesis, we observed a decrease in heterotypic DENV-2 neutralizing antibodies over time. Like Kochel et al. (*17*), we were unable to detect significant heterotypic neutralization to the Asian genotype DENV-2 viruses. We did not observe increases in heterotypic DENV-3 or DENV-4 antibody titers. Unexpectedly, we did observe an increase in titer of homologous DENV-1 antibodies at 4–8 and 20–22 years after infection.

Although we did not detect significant heterotypic neutralization of the A15/81 Asian DENV-2 strain at a 1:10 dilution, these results do not rule out the possibility that neutralization might have been detected at lower dilutions. Limited serum volumes prevented us from testing the panel of DENV-1–immune serum collected during 1981–1985 for heterotypic neutralizing antibodies to the 1997 DENV-2 strain (58/97) isolated during the 1997 Santiago de Cuba epidemic and classified as Asian genotype (*9*). However, we did test group 2 serum (collected in 1999). No differences were observed in neutralization of the A15/81 or 58/97 DENV-2 strains by DENV-1–immune serum at a dilution of 1:10. Only 18% of group 2 samples showed neutralizing activity to the 58/97 DENV-2 strain with a geometric mean titer of 7.1. We believe the antibodies measured in the 2 groups were derived from infections that occurred in Cuba in 1977. Among persons who contributed to each group of serum samples, none had been vaccinated against yellow fever and few had traveled outside Cuba.

Our results demonstrate long-term changes in heterotypic dengue neutralizing antibodies. Although we did not detect neutralization of Asian DENV-2 in vitro, we question whether some degree of neutralization might have occurred in vivo, which might have affected disease severity. For example, the neutralization test is not particularly good at predicting protective immunity. Recently, Endy et al. (*26*) reported that levels of preinfection neutralizing antibodies against DENV-2 (standard strain and virus isolated during illness) were not associated with severity of secondary DENV-2 infection. However, in the same study, higher levels of preexisting neutralizing antibodies against DENV-3 were associated with lower viremia levels and milder disease. Many possible reasons exist for these complexities, including the artificiality of existing dengue viral neutralization tests or differences in ability of antibodies to neutralize different dengue strains of the same genotype.

The first report that heterotypic neutralizing antibodies might be an important mechanism of down-regulating the severity of dengue infection was deduced from the prospective study of school children in Bangkok, Thailand. Children who had heterotypic DENV-2 neutralizing antibodies before they became infected with DENV-2 (their antibodies were predominantly the result of prior DENV-1 infections) experienced only inapparent secondary DENV-2 infections. In contrast, DHF/dengue shock syndrome developed in children whose serum lacked detectable heterotypic neutralizing antibodies (but contained dengue-enhancing antibodies) (*15*). These studies tested undiluted serum, before illness, in elutriated monocytes from donors with no immunity to flaviviruses.

The contemporary explanation of long-term persistence of antibodies after viral infection is based on evidence of the presence of long-lived B memory and plasma cells. The improvement in homotypic neutralizing antibody titer and decrease in heterotypic neutralizing antibody titer described here is reminiscent of affinity maturation. However, long-lived plasma cells would not be expected to participate in the selection process required for affinity maturation. In an earlier study of serum samples from US military personnel with inapparent Japanese encephalitis virus infection, over the 1–5 years after infection, the log neutralization index increased from a mean of 1.7 to 3.5 (*27*). This earlier study and our present study are unique in that they measured qualitative attributes of human antibodies for long intervals after infection with wild-type flavivirus. Our preliminary data suggest a continuous process of selection of populations of dengue virus antibodies with increasing homologous reactivity and a concurrent decrease in heterotypic cross-reactions.

Our results require confirmation and further study. To study antibody titers in the same persons, we will attempt to locate the persons from whom samples were collected during 1981–1985 and collect serum in volumes that may permit tests for dengue-enhancing antibodies and neutralizing antibodies to several dengue strains. Our present results could simply reflect increases and decreases in avidity of antibodies with the passage of time. We plan to

investigate this possibility by using the same strains as well as a nondengue flavivirus antigen.

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Dr Guzman is head of the virology department at the Tropical Medicine Institute in Havana. Dengue is her main subject of investigation.

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Address for correspondence: Maria G. Guzman, “Pedro Kourí” Tropical Medicine Institute, Autopista Novia del Mediodía, Km 6, P.O. Box Marianao 13, Ciudad Habana, Cuba; email: lupe@ipk.sld.cu

Waterborne Toxoplasmosis, Northeastern Brazil

Jorg Heukelbach,*† Vanessa Meyer-Cirkel,‡ Rômulo César Sabóia Moura,§ Marcia Gomide,* José Ajax Nogueira Queiroz,* Peter Saweljew,¶ and Oliver Liesenfeld‡

Two waterborne outbreaks of toxoplasmosis have been described recently in southern Brazil. We present data from a community-based study of pregnant women in northeastern Brazil. Consumption of homemade ice was the only variable associated with seropositivity (adjusted odds ratio, 3.1, 95% confidence interval, 1.53–6.24). Our results suggest that water is a source of infection with *Toxoplasma gondii*.

Toxoplasma gondii is usually transmitted by consumption of food or water contaminated with oocysts from cat feces or soil or by eating undercooked meat that contains oocysts (*1*,*2*). Data from Canada and southern Brazil indicate that infection also occurs by drinking unfiltered water contaminated with oocysts (*3*–*6*). In Latin America, seroprevalence of immunoglobulin G (IgG) to *T. gondii* is generally high and ranges from 51% to 72% (*2*). In Brazil, factors predisposing for infection with *T. gondii* are not completely understood, and relatively little is known about the epidemiology of toxoplasmosis.

The Study

This community-based cross-sectional study was undertaken in Cascavel Municipality, a typical semirural municipality ≈70 km south of Fortaleza, the capital of Ceará State in northeastern Brazil. In 2003, Cascavel had a population of 57,000. The main sources of income are fishing, agriculture, tourism, and the cashew nut industry.

Most (95%) of the pregnant women in Cascavel are registered in the Family Health Program and receive prenatal care from the public health system. All pregnant women at <26 weeks of gestation registered in this program from May to August 2003 were visited at home and asked to participate in the study. One female investigator interviewed the women with respect to demographic, socioeconomic status, and behavioral characteristics by using pretested structured questionnaires. Emphasis was given to the presence or ownership of animals, eating habits, soil contact and drinking water sources. The questionnaire was adapted from a study conducted in southern Brazil (*3*).

Serum samples were tested for IgM and IgG antibodies to toxoplasma by ELISA (Vidas, bioMérieux, Nürtingen, Germany). Women with positive IgG titers but negative IgM titers were considered latently infected. Women with positive titers both for IgG and for IgM were considered to possibly have recent infections and were further tested for avidity of IgG antibodies (Vidas, bioMérieux). Sensitivity and specificity of the Vidas test are 97.3% and 99.8, respectively (*7*). We were unable to rule out recent infections in women with low or intermediate IgG avidity.

Ethical approval for the study was obtained from the ethical review board of Cascavel Municipality. Before the study, community meetings were held in which the objectives of the study were explained. Informed written consent was obtained from all study participants. Women with possible recent infections and their newborns received free therapy and medical assistance.

Data were entered twice into a database by using EpiInfo version 6.04d software (Centers for Disease Control and Prevention, Atlanta, GA, USA) and checked for errors. Multivariate logistic regression with backward elimination was used to calculate adjusted odds ratios for the independent association between toxoplasma infection (defined as the presence of specific IgG antibodies) and possible risk factors. For logistic regression analysis, STATA version 7 software (Stata Corporation, College Station, TX, USA) was used.

A total of 231 pregnant women were identified during the study period, and all agreed to participate (median age 23 years, range 14–43 years). Of these women, 161 (69.7%, 95% confidence interval [CI] 63.3–75.6) had IgG antibodies against *T. gondi*. A total of 68% of women <25 years of age were seropositive. Prevalence was not significantly higher in older women than in women <25 years of age ($p = 0.5$). Five women (2.2%, 95% CI 0.7–5.0) had IgM antibodies; of these women, 3 (60%) had low-avidity IgG antibodies.

Bivariate analysis for factors associated with *T. gondii*–specific IgG showed that none of the demographic or socioeconomic variables were associated with infection. Other risk factors previously described, such as contact with cats or consumption of raw meat, were not associated with IgG seropositivity. In the logistic regression model, the only variable associated with IgG antibodies to toxo-

*Federal University of Ceará School of Medicine, Fortaleza, Ceará, Brazil; †James Cook University, School of Public Health, Tropical Medicine, and Rehabilitation Sciences, Townsville, Queensland, Australia; ‡Charité Medical School, Berlin, Germany; §Mandacaru Foundation, Fortaleza, Ceará, Brazil; and ¶bioMérieux, Nürtingen, Germany

plasma was regular consumption of homemade ice (Table). Four (80%) of the 5 IgM-positive women regularly consumed these ices. This ice is made by people at home, is sold locally, and consists of tap water, artificial flavor, and sugar, frozen in small plastic bags.

Using a commercial extraction kit (Qiagen, Valencia, CA, USA), we extracted DNA from randomly chosen aliquots (1.5 mL) of >50 homemade ice samples obtained from local vendors in Cascavel and performed a standardized nested PCR assay (*T. gondii* B1 gene, sensitivity 1 parasite). Toxoplasma-specific DNA was not detected in any of these samples.

Conclusions

The IgG prevalence of 70% found in this study is consistent with results of a study in Fortaleza in which 72% of pregnant and postpartum women were seropositive for IgG to toxoplasmosis (*8*). In our study population, prevalence did not increase with age, which indicated that in this setting most infections occur in childhood or adolescence. Only 2% of our study population had *T. gondii*–specific IgM antibodies.

Risk factors identified in other studies were not associated with toxoplasmosis in the typical semirural community in our study. A previous study from Brazil reported an outbreak of toxoplasmosis associated with the consumption of raw mutton (*9*). Other studies from south Brazil suggested that consumption of undercooked beef and working in a garden were risk factors (*3*,*10*). We did not find an association between consumption of raw meat and seropositivity, which may be because people in northeastern Brazil (unlike those in southern Brazil) do not eat undercooked or raw meat. None of our study participants reported eating undercooked meat.

Ownership of free-ranging chickens and consumption of cheese were negatively associated with toxoplasmosis. These 2 variables are associated with higher socioeconomic status in rural communities in northeastern Brazil. We cannot rule out that our results were confounded, even when we used a score to quantify socioeconomic status in multivariate regression analysis.

We found that homemade ice, which is stored in small plastic bags, was a possible risk for infection. However, toxoplasma DNA was not detectable in any of the ice samples. This finding does not rule out that the water was contaminated because identification of parasites in water requires large volumes. Drinking water (which is used for the preparation of this type of ice) was not a risk factor for infection. We cannot rule out the possibility that the outer surface of the plastic bags in which the ice was packed was contaminated by oocysts from soil. These plastic bags are often opened by ripping them with the teeth, which may result in infection. Our results confirm the findings of Bahia-Oliveira et al. (*3*), who identified a marginal association between consumption of homemade ice stored in plastic bags and *T. gondii* infection.

Because our study was community based and included virtually all women who were pregnant during the study period, the results are highly representative for the pregnant population. However, our study has limitations. Because of the cross-sectional design, causal and temporal relationships are difficult to establish. Additionally, because few women were IgM positive, IgG positivity was used as a marker for toxoplasma infection. However, because IgG antibodies to toxoplama persist for years, many infections had probably been acquired some years ago, the environment and behavior patterns may have changed, and risks that are no longer present would not have been included.

In conclusion, toxoplasma infection in the study area was high in pregnant women. The study indicates that the pattern of risk factors for infection is different from that found in other studies. Future studies should show if these results are caused by chance or unknown confounders, or if the consumption of homemade ice has a direct association with infection with *T. gondii*.

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Table. Multivariate logistic regression analysis of factors associated with infection with *Toxoplasma gondii* in pregnant women, northeastern Brazil

Independent variable	Adjusted odds ratio	95% Confidence interval	p value
Regular consumption of homemade ice	3.10	1.53–6.24	0.002
Having feral cats in yard	1.72	0.85–3.47	0.13
Being of low socioeconomic status*	0.94	0.77–1.14	0.5
Living on an unpaved street	0.50	0.23–1.07	0.07
Free-ranging chickens in yard	0.40	0.19–0.81	0.01
Consumption of cow milk	0.42	0.16–1.10	0.08
Consumption of cheese	0.47	0.25–0.90	0.02
Consumption of ice cream	0.59	0.31–1.11	0.10
Consumption of chicken	0.22	0.057–1.30	0.10

*According to an ordinal socioeconomic score from 0 to 10.

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Dr Heukelbach is professor of epidemiology at the Federal University of Ceará, Ceará, Brazil. His research interests include the epidemiology and control of infectious and parasitic diseases.

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Address for correspondence: Jorg Heukelbach, Departamento de Saúde Comunitária, Faculdade de Medicina, Universidade Federal do Ceará, Rua Prof. Costa Mendes 1608, 5. Andar, Fortaleza, Ceará, CE 60430-140, Brazil, email: heukelbach@web.de

Avian Influenza Risk Perception, Europe and Asia

Onno de Zwart,* Irene K. Veldhuijzen,* Gillian Elam,† Arja R. Aro,‡ Thomas Abraham,§ George D. Bishop,¶ Jan Hendrik Richardus,# and Johannes Brug#

During autumn 2005, we conducted 3,436 interviews in European and Asian countries. We found risk perceptions of avian influenza to be at an intermediate level and beliefs of efficacy to be slightly lower. Risk perceptions were higher in Asia than Europe; efficacy beliefs were lower in Europe than in Asia.

The possibility of an influenza pandemic presents a major public health challenge. Since 2003, outbreaks of avian influenza (AI) have occurred in Asian, European, and African countries. As of August 21, 2006, the total number of cases was 240 and the number of deaths was 141 (*1*). A crossover of current human influenza virus with the avian H5N1 virus could result in a virus capable of human-to-human transmission and the start of a new pandemic.

Despite extensive media attention for avian influenza, knowledge about risk perception of AI is scarce. We therefore explored the conditions for effective nonmedical interventions. If an influenza pandemic occurs, public health authorities will be dependent on the willingness and ability of the public to adhere to recommendations regarding personal hygiene, vaccination and prophylaxis, quarantine, travel restrictions, or closing of public buildings (*2*,*3*). Adherence, however, cannot be assumed. Evaluation of the outbreak of H7N7 AI in the Netherlands in 2003 showed that adherence to antiviral therapy and behavioral measures, such as wearing face masks and goggles, was low (*4*).

Our ability to promote health-protective behavioral change depends on our knowledge of determinants of such behavior (*5*). The protection motivation theory posits that health-protective actions are influenced by risk perceptions (*6*–*8*). Risk perceptions are defined by the perceived seriousness of a health threat and perceived personal vulnerability. However, the protection motivation theory explicitly states that higher risk perceptions will only predict protective behavior when people believe that effective protective actions are available (response efficacy) and that they have the ability to engage in such protective actions (self-efficacy).

*Municipal Public Health Service, Rotterdam, the Netherlands; †Health Protection Agency—Centre for Infections, London, United Kingdom; ‡University of Southern Denmark, Esbjerg, Denmark; §University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China; ¶National University of Singapore, Singapore; and #Erasmus University Medical Center Rotterdam, Rotterdam, the Netherlands

The Study

We investigated risk perceptions and efficacy beliefs related to AI of a random sample of persons in 8 areas. Random digital dialing was used to select the samples, and data were collected by using computer-assisted telephone interviewing. Interviews were conducted from September 20 through November 22, 2005, in 5 European countries (Denmark, the Netherlands, United Kingdom, Spain, and Poland) and 3 East Asian areas (Singapore; Guangdong Province, People's Republic of China; and Hong Kong, Special Administrative Region, People's Republic of China). At the time the telephone survey was conducted, on October 14, 2005, the media announced the introduction of AI in Europe. We therefore ensured that at least 90 interviews were conducted in each country after October 18, 2005. The questionnaire focused on risk perception of AI and other infectious diseases, precautionary behavior, and use of information sources; it was based on our earlier study of risk perception of severe acute respiratory syndrome (SARS) (*9*). Respondents first received a brief explanation of AI.

In line with the protection motivation theory (*8*), a measure of risk perception was constructed by multiplication of seriousness (scale 1–10) and vulnerability (scale 1–5). To make the scores comparable, the seriousness score was first divided by 2. To normalize the skewed distribution of the new variable, a square-root transformation was performed, which resulted in a measure of risk perception on a scale from 1 (low) to 5 (high).

A total of 3,436 respondents were interviewed; participation rates varied from 12.9% in Asia to 81.1% in Poland. Most respondents were female (Table 1). European respondents were significantly older than Asian respondents (mean age 47 and 39 years, respectively, range 18–75 years, $t = 16.2$; degrees of freedom [df] = 3,351; $p<0.001$). Overall, 45% of respondents thought they were likely or very likely to become infected with AI if an outbreak occurred in their country. This perception varied from 32% in Denmark and Singapore to 61% in Poland and Spain. Risk perception scores varied significantly across countries; the highest mean score was in Poland and the lowest was in Denmark (Table 2). Higher scores were observed in Europe than in Asia ($t = 5.2$; df = 3,250; $p<0.001$), and differences between individual countries within Europe were significant. Multivariate analysis showed that country, sex, and age group remained inde-

Table 1. Distribution of general characteristics of the study population, by country or region, September 20–November 22, 2005*

Characteristic	No. (%)										
	DNK	POL	NLD	UK	ESP	CHN	HKG	SGP	Europe	Asia	Total
Total	463 (14)	488 (14)	400 (12)	401 (12)	425 (12)	404 (12)	396 (12)	426 (13)	2,177 (64)	1,226 (36)	3,403 (100)
Sex											
Male	40	39	42	41	41	47	44	43	40	45	42
Female	60	61	58	59	59	53	56	57	60	55	58
Age group, y											
18–30	13	18	10	13	17	43	27	35	14	35	22
31–44	31	31	31	35	34	34	35	31	32	33	33
45–60	36	32	37	31	32	19	27	20	33	22	29
61–75	20	19	24	21	17	4	12	14	20	10	16
Area											
City	26	21	9	20	45	86	90	81	24	86	46
Town	38	25	37	45	42	9	4	16	37	10	27
Village/ countryside	37	54	55	36	13	4	6	2	39	4	26
Education											
Primary or less	17	8	5	2	22	4	13	3	11	7	9
Low	31	22	28	20	9	19	20	11	22	16	20
Intermediate	38	43	35	35	31	35	32	38	37	35	36
High	13	28	32	43	38	42	35	48	30	42	34

*DNK, Denmark; POL, Poland; NLD, the Netherlands; UK, United Kingdom; ESP, Spain; CHN, China; HKG, Hong Kong; SGP, Singapore.

pendent significant factors and showed a significant interaction between country and sex and between country and age group (Figure). In all countries, except Singapore, risk perception was higher among women than men, but this difference was smaller in Asian than in European countries. The effects of age also varied by country; mean risk perception levels were higher in older age groups in Europe but not in Asia.

Response efficacy and self-efficacy also varied across countries; levels were highest in China and lowest in the Netherlands (Table 2). Mean response efficacy and self-efficacy were significantly higher in Asia than in Europe (response efficacy $t = -14$; $df = 2,868$; $p<0.001$; self-efficacy $t = -20$; $df = 2,701$; $p<0.001$). Response and self-efficacy were inversely associated with risk perception ($p = 0.013$ and $p<0.001$, respectively).

Multivariate analysis also showed that country, but not sex or age, was significantly associated with response efficacy. Country, sex, and age group were all significantly associated with self-efficacy. Self-efficacy levels were lower for women compared with men and for the youngest age group compared with older respondents. Risk percep-

Table 2. Perceived risk perception and efficacy beliefs with regard to a potential influenza outbreak, September 20–November 22, 2005*

Country or region	Mean score (95% CI)				
	Scale 1–10	Scale 1–5			
	Seriousness	Vulnerability	Risk perception†	Response efficacy	Self-efficacy
DNK	6.08 (5.83–6.33)	2.82 (2.71–2.92)	2.73 (2.65–2.81)	2.32 (2.23–2.41)	2.15 (2.06–2.24)
POL	7.49 (7.29–7.70)	3.43 (3.31–3.54)	3.48 (3.39–3.57)	2.55 (2.46–2.64)	2.06 (1.96–2.16)
NLD	7.67 (7.48–7.87)	3.17 (3.07–3.27)	3.40 (3.32–3.48)	2.25 (2.14–2.35)	1.74 (1.66–1.83)
UK	7.38 (7.16–7.61)	2.93 (2.81–3.05)	3.17 (3.07–3.26)	2.41 (2.32–2.51)	2.03 (1.93–2.12)
ESP	6.76 (6.53–6.99)	3.43 (3.32–3.53)	3.29 (3.20–3.37)	2.75 (2.65–2.85)	2.26 (2.15–2.36)
CHN	6.58 (6.33–6.82)	2.88 (2.76–2.99)	2.94 (2.85–3.04)	2.99 (2.92–3.06)	2.90 (2.82–2.99)
HKG	7.02 (6.81–7.23)	3.33 (3.23–3.42)	3.33 (3.25–3.40)	2.95 (2.87–3.03)	2.64 (2.55–2.73)
SGP	6.63 (6.35–6.91)	2.70 (2.57–2.83)	2.82 (2.71–2.93)	2.81 (2.71–2.91)	2.70 (2.61–2.80)
Europe‡	7.06 (6.96–7.16)	3.16 (3.11–3.21)	3.21 (3.17–3.25)	2.46 (2.41–2.50)	2.05 (2.01–2.10)
Asia	6.74 (6.60–6.88)	2.97 (2.90–3.03)	3.03 (2.97–3.08)	2.92 (2.87–2.96)	2.75 (2.69–2.80)
Total	6.95 (6.86–7.03)	3.09 (3.05–3.13)	3.14 (3.11–3.17)	2.63 (2.59–2.66)	2.31 (2.27–2.34)

*CI, confidence interval; DNK, Denmark; POL, Poland; NLD, the Netherlands; UK, United Kingdom; ESP, Spain; CHN, China; HKG, Hong Kong; SGP, Singapore.
†Square root of the multiplication of seriousness divided by 2 and vulnerability.
‡Differences in mean scores between Europe and Asia are significant for all measures ($p<0.001$).

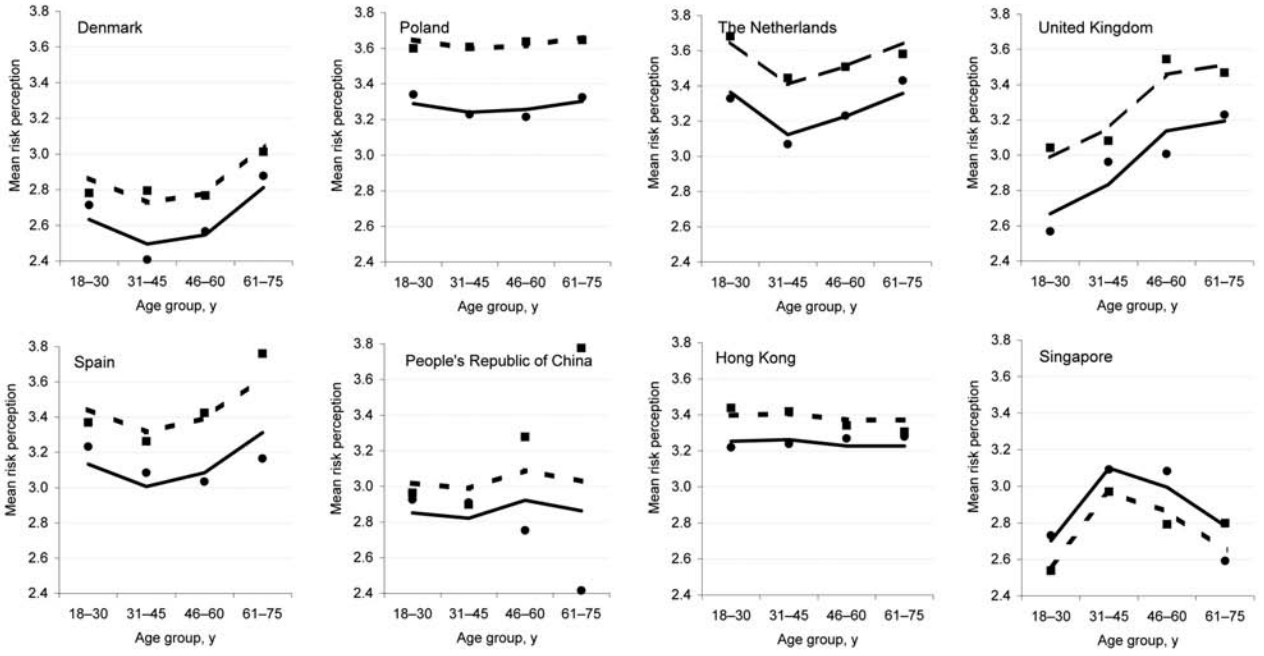

Figure. Mean risk perception by country or region, sex, and age group. Lines, predicted means; squares and circles, observed means; solid line and circles, male; dashed line and squares, female.

tion and efficacy levels before and after the introduction of avian influenza in Europe did not differ significantly.

Conclusions

Our study showed that risk perceptions for AI appear to be at an intermediate level and that efficacy beliefs are slightly lower. Both differ according to country or region. No evidence was found that the introduction of AI in Europe in October 2005 influenced perceptions of risk or efficacy.

Fielding et al. have reported on risk perception of AI in Hong Kong with a focus on live chicken sales (*10*). Although our results are difficult to compare with theirs, our study appears to indicate a higher feeling of vulnerability, with 41.8% of Hong Kong respondents thinking it likely or very likely that they would become infected with influenza during an outbreak. Takeuchi's interviews on food safety practices of consumers in Thailand found high levels of knowledge of AI but lower levels of risk perception and behavior change (*11*). If we compare our results with those from several studies on perception of risk for SARS, we find that perception of risk for SARS in some of the Asian countries was relatively low compared with that in the United States (*12*). In the Netherlands, however, perception of risk for SARS was low, whereas our present study indicates that it is high for influenza (*9*).

The lower level of risk perception for AI in Asia may be related to the proximity to the current outbreak and the experience with the SARS epidemic. These experiences may have resulted in the notion that new epidemics of infectious diseases can be controlled. Also, despite the fact that the first cases of H5N1 influenza among humans in Asia were reported in 2003, a larger outbreak did not ensue. Accordingly, risk perception research has shown that the public may be more optimistic when familiar risks are perceived to be largely under volitional control (*13*,*14*).

Our study has several implications for public health policy and research. Although in all countries an influenza pandemic is perceived as a real risk, the level of self-efficacy appears to be rather low. When developing preparedness plans for an influenza pandemic, specific attention should therefore be paid to risk communication and how perceived self-efficacy can be increased; otherwise, adherence to preventive measures may be low.

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Mr de Zwart is head of the Division of Infectious Diseases Control of the Municipal Public Health Service Rotterdam Area and PhD candidate at the Department of Public Health, Erasmus University Medical Center Rotterdam. His research interests focus on risk perception and communication in relation to infectious diseases and development and evaluation of preventive

interventions for HIV, sexually transmitted infections, and viral hepatitis.

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Address for correspondence: Onno de Zwart, Municipal Public Health Service Rotterdam Area, Division of Infectious Disease Control, PO Box 70032, 3000 LP Rotterdam, the Netherlands; email: dezwarto@ggd.rotterdam.nl

No Evidence of Avian Influenza A (H5N1) among Returning US Travelers

Justin R. Ortiz,* Teresa R. Wallis,* Mark A. Katz,* LaShondra S. Berman,* Amanda Balish,* Stephen E. Lindstrom,* Vic Veguilla,* Kathryn S. Teates,* Jacqueline M. Katz,* Alexander Klimov,* and Timothy M. Uyeki*

We reviewed reports to the Centers for Disease Control and Prevention of US travelers suspected of having avian influenza A (H5N1) virus infection from February 2003 through May 2006. Among the 59 reported patients, no evidence of H5N1 virus infection was found; none had direct contact with poultry, but 42% had evidence of human influenza A.

As of June 2006, the epizootic of highly pathogenic avian influenza A (H5N1) virus among birds had spread to 3 continents (*1*). Sporadic human H5N1 cases characterized by severe respiratory disease with high case-fatality have been reported in 10 countries: Azerbaijan, Cambodia, Djibouti, Egypt, Indonesia, Iraq, People's Republic of China, Thailand, Turkey, and Vietnam (*2*). Investigations have implicated direct contact with diseased poultry as the primary risk factor for H5N1 virus infection (*3*,*4*).

To date, highly pathogenic H5N1 virus infections among poultry or wild birds in the United States have not been identified. However, US residents may be exposed if they travel to H5N1-affected countries. In February 2003, the Centers for Disease Control and Prevention (CDC) developed interim guidance for testing of suspected cases of H5N1 in returned travelers (*5*). CDC revised the recommendations in February 2004 (*6*). We report the results of investigations of patients with suspected H5N1 that were reported to CDC from February 2003 through May 2006.

The Study

We retrospectively analyzed available data on US patients with suspected H5N1 virus infection that were reported to CDC by clinicians and public health departments from February 2003 through May 2006. Clinical and epidemiologic data about reported patients were communicated to CDC by telephone, email, and/or fax. For each patient, we assessed whether criteria for recommended H5N1 testing were met (suspected H5N1 case definition). The suspected H5N1 case definition had 2 components: the hospitalized case definition included severe respiratory illness and recent travel to an H5N1-affected country; and the ambulatory case definition included acute respiratory illness, contact with domestic poultry or a known or suspected H5N1 case-patient, and recent travel to an H5N1-affected country (*6*) (Figure). Contact was defined as proximity ≤1 m, and direct contact was defined as physical touching.

If a patient met the suspected H5N1 case definition, or if exposure data were incomplete and clinicians or public health authorities had persistent concerns, H5N1-specific testing was recommended by CDC. A standard case report form was completed by state health departments.

Diagnostic testing for patients with suspected H5N1 virus infection was performed at CDC, state laboratories, or both. Procedures for reverse transcription–PCR (RT-PCR) and microneutralization assay for H5N1 have been previously described (*7*,*8*). Epidemiologic and laboratory data were analyzed by using EpiInfo version 3.3.2 (CDC, Atlanta, GA, USA).

Fifty-nine patients from 26 states were reported to CDC for suspicion of H5N1 virus infection from February 2003 through May 2006 (Table and Figure). Nineteen (37%) were male (n = 52), and the median age was 47 years (n = 49, range 2–87 years). Of the samples received from 37 patients that were tested at CDC, none had evidence of H5N1 virus infection. CDC tested samples from 8 patients for human influenza A only, and 5 were positive. Among the samples from 22 (37%) patients not tested at CDC, 4 (7%) were tested for H5N1 at state public health laboratories, and all were negative. Of the remaining 18 (31%) patients, 15 were not tested for H5N1 and state testing data were unavailable for 3 (Figure).

On the basis of available information, 27 (46%) patients met the CDC suspected H5N1 case definition (Table). Fourteen (24%) had severe, acute respiratory illness with recent travel to an H5N1-affected country. Overall, 25 (42%) patients, including 2 of 4 who died, tested positive for human influenza A virus infection. In addition, 52% of the 27 patients who met the CDC suspected H5N1 case definition had samples that tested positive for human influenza A. Four influenza A cases occurred outside the US influenza season. Of the influenza A–positive patients, 10 had H3N2 viral isolates that were characterized at CDC. All isolates were similar to human influenza A virus strains concurrently circulating in North America.

Other diagnoses included community-acquired pneumonia, bronchiolitis obliterans and organizing pneumonia,

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Table. Characteristics of cases referred to CDC for assessment of H5N1 virus infection*

Case-patient characteristics (n = 59)	No. (%)
Met CDC suspect H5N1 case definition	27 (46)
Met hospitalized case definition criteria	14 (24)
Met ambulatory case definition criteria	13 (22)
Bird proximity ≤1 m	14 (24)
Direct bird contact	0
Contact with confirmed human H5N1 case	0
Onset outside US influenza season	7 (12)
Outcome	
Hospitalized	20 (34)
Deceased	4 (7)
Diagnosis†	
No	28 (47)
Negative influenza test result but ILI and influenza A (H3)–positive contact	8 (14)
Human influenza A (H3)	23 (39)
Human influenza A (H1N1)	1 (2)
Human Influenza A by rapid antigen test‡	1 (2)
Community-acquired pneumonia	2 (3)
Bronchiolitis obliterans and organizing pneumonia	1 (2)
Lymphoma	1 (2)
Rickettsial typhus	1 (2)
Toxic shock syndrome	1 (2)
Country visited§¶	
People's Republic of China	21 (36)
Vietnam	18 (31)
Thailand	11 (19)
South Korea	5 (8)
Taiwan	3 (5)
Cambodia	2 (3)
Other#	6 (10)
No foreign travel	4 (7)
Unknown travel history	2 (3)

*CDC, Centers for Disease Control and Prevention; ILI, influenzalike illness.
†Sum of percentages >100% due to rounding.
‡Clinical diagnosis of human influenza. The patient was reported to CDC for suspected H5N1 virus infection but was determined to lack risk factors to warrant H5N1-specific testing.
§Not mutually exclusive.
¶As of June 15, 2006, highly pathogenic avian influenza A (H5N1) has not been confirmed by the World Health Organization or World Organization for Animal Health (OIE) in Saipan, Saudi Arabia, Singapore, and Taiwan (*1*). Japan was declared free of H5N1 by OIE on July 12, 2004 (*1*), 13 mo before the visit by a reported patient. South Korea was declared free of H5N1 (*1*) at the time of a visit by a reported patient.
#One each for Indonesia, Japan, Malaysia, Saipan, Singapore, and Saudi Arabia.

toxic shock syndrome, lymphoma, and rickettsial typhus (Table). Among 28 patients without a diagnosis, 8 (29%) tested negative for influenza but had influenzalike illness and contact with an influenza A (H3)–positive person.

Among all reported patients, 52 (88%) had traveled to ≥1 of 11 countries in Asia with either confirmed human H5N1 cases or H5N1 in avian species before illness onset (Table). Four (7%) patients with suspected H5N1 virus infection had not traveled outside the United States, but they had contact with recent travelers to Asia, and 1 had traveled to a country without confirmed H5N1 in poultry or wild birds. Although 14 (24%) reported having been within ≤1 m of any live poultry or domesticated birds in Asia, none reported touching live poultry, domesticated birds, or recently butchered poultry. No patients with suspected H5N1 virus infection had contact with any confirmed or suspected human H5N1 case-patients.

Conclusions

Our review of patients evaluated for H5N1 virus infection among returned US travelers through May 2006 indicates that the risk of H5N1 to US travelers has been extremely low to date. A high proportion of the reported patients had evidence of human influenza A virus infection, but none tested positive for H5N1. Although direct contact with infected poultry is the primary risk factor for H5N1 virus infection (*3*,*4*), H5N1 virus transmission has been low, even among persons directly exposed to poultry suspected of infection with currently circulating H5N1 virus strains (*9*,*10*). None of the patients reported to CDC had touched poultry, and 48% of persons with cases that met the CDC suspected H5N1 case definition had not been within ≤1 m of birds during travel.

Our finding that 42% of patient with suspected H5N1 virus infection had human influenza A emphasizes the importance of considering this disease year-round in returned travelers with acute respiratory infections. Human influenza activity in tropical and subtropical countries occurs year-round outside the typical US influenza season (*11*) and is the most frequent vaccine-preventable infection among travelers from Europe to tropical and subtropical countries (*12*). Moreover, the effect of influenza disease in tropical countries is substantial; for example, rates of influenza-associated hospitalizations in subtropical Hong Kong approximate US estimates (*13*).

Annual influenza vaccination is the best way to prevent human influenza. Influenza vaccine effectiveness depends upon multiple factors, including the degree of similarity between the vaccine strains and those in circulation. Persons at high risk for complications from influenza who were not vaccinated during the preceding fall or winter should consider influenza vaccination, if available, before travel (*14*). However, no human H5N1 vaccine is currently available.

Our study is subject to several limitations. The reported patients may not be representative of all US travelers at risk for H5N1 virus infection among whom respiratory illness developed, but were persons for whom health departments and physicians sought CDC consultation. States may have evaluated travelers for H5N1 virus infection without notifying CDC. Additionally, some reported patients were not tested for H5N1 if available epidemiologic and clinical information suggested that H5N1 virus infection was unlikely. Clinical charts were not

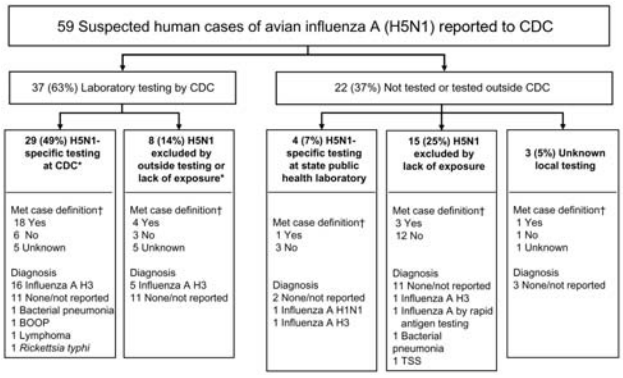

Figure. Influenza testing of suspected US cases of avian influenza A (H5N1) reported to the Centers for Disease Control and Prevention (CDC) from February 2003 through May 2006. *Of the 37 samples tested by CDC, 35 were respiratory samples, 1 was serum, and 1 was a lung specimen. All 35 respiratory samples received by CDC were tested for human influenza by reverse transcription-PCR, and the serum sample was tested by microneutralization assay. †CDC suspected H5N1 case definition, February 2, 2004–June 7, 2006 (*6*): a patient is hospitalized and has radiographically confirmed pneumonia, acute respiratory distress syndrome, or other severe respiratory illness for which an alternate diagnosis has not been established; and the patient has a history of travel within 10 days of symptom onset to a country with documented H5N1 avian influenza in poultry and/or humans; or a patient is hospitalized or ambulatory and has a documented temperature >38°C (>100.4°F); and has a cough, sore throat, or shortness of breath; and has a history of contact with domestic poultry or a patient with known or suspected H5N1 case in an H5N1-affected country <10 days of symptom onset. BOOP, bronchiolitis and obliterans organizing pneumonia; TSS, toxic shock syndrome.

independently reviewed, and clinical, epidemiologic, and laboratory data were limited to that sent to CDC by state and local health departments. In many instances, the role of CDC was to exclude the diagnosis of H5N1, and further testing to establish a diagnosis other than influenza was not always performed.

Continued surveillance and testing for H5N1 is warranted, given the current H5N1 epizootic, the ongoing occurrence of human H5N1 cases globally, and the importance of identifying influenza A viruses with pandemic potential in the United States as early as possible. In 2006, CDC and WHO revised their definitions for suspected H5N1 cases (*15*,*16*). The revised CDC suspected H5N1 case definition now specifically requires that case patients have touched poultry or have had contact with a patient with confirmed or suspected H5N1 virus infection. It also more clearly defines an H5N1-affected country. The findings of our study support these changes. Guidance for the evaluation of patients with suspected H5N1 virus infection should continue to be evaluated as more epidemiologic data become available.

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Dr Ortiz is an Epidemic Intelligence Service Officer in the Influenza Division at CDC. His primary research interest is the epidemiology of viral respiratory infections.

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Address for correspondence: Justin R. Ortiz, Influenza Division, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A32, Atlanta, GA 30333, USA; email: dzv3@cdc.gov

Postpartum Mastitis and Community-acquired Methicillin-resistant *Staphylococcus aureus*

Pavani Reddy,* Chao Qi,* Teresa Zembower,* Gary A. Noskin,* and Maureen Bolon*

This single-center, case-control study documents a relative increase in methicillin resistance among 48 cases of *Staphylococcus aureus*–associated postpartum mastitis during 1998–2005. Of 21 cases with methicillin resistance, 17 (81%) occurred in 2005. Twenty (95%) isolates contained the *Staphylococcus* cassette chromosome *mec* type IV gene; this suggests that the increase is due to community-acquired methicillin-resistant *S.aureus*.

Postpartum mastitis (PPM) occurs in as many as one third of breastfeeding women in the United States and leads to breast abscess formation in ≈10% of cases (*1*,*2*). Although breast milk cultures are not routine in PPM management, the growth of potentially pathogenic bacteria (such as β-hemolytic streptococci or *Staphylococcus aureus*) is associated with longer time to recovery and more frequent abscess formation (*3*). *S. aureus* is the most common bacterium isolated from such cultures, representing 37%–50% of isolates (*4*,*5*).

Reports of methicillin-resistant *S. aureus* (MRSA) PPM among young, healthy women lacking traditional risk factors for MRSA have emerged in the past few years (*6*,*7*). Isolates in these cases of community-acquired infection (CA-MRSA) remain susceptible to multiple non–β-lactam antibiotics and possess distinct molecular features (*8*).

Although risk factors associated with skin and soft tissue infections due to CA-MRSA have been described (*8*,*9*), characteristics unique to patients with CA-MRSA PPM are unknown. To identify risk factors, complications, and outcomes among patients with CA-MRSA PPM, we conducted a retrospective, case-control study to include all *S. aureus*–associated cases at a single institution over an 8-year period. MRSA isolates were analyzed by PCR for the presence of the *Staphylococcus* cassette chromosome (SCC) *mec* type IV gene, which is commonly associated with community-acquired infection.

The Study

We considered for analysis all patients from Northwestern University's Prentice Women's Hospital and affiliated Lynn Sage Comprehensive Breast Center with wound, fluid, drainage, or breast milk cultures positive for *S. aureus* from January 1998 through December 2005. Case-patients were defined as patients with PPM and a corresponding culture positive for MRSA. Control-patients were defined as patients with PPM and a corresponding culture positive for methicillin-susceptible *S. aureus* (MSSA). Patients who had no evidence of mastitis or who had a history of MRSA were excluded from the study. SCC*mec* types I–V were identified by a PCR-based multiplex assay; rapid bacterial DNA extraction and PCR amplification were performed as described elsewhere (*10*).

Forty-eight cases of *S. aureus*–associated PPM were identified during the study period; 21 cases were due to MRSA and 27 cases were due to MSSA. A relative increase in MRSA PPM was noted in the later years of the study (Figure 1, p = 0.04). MRSA and MSSA patients did not differ significantly with respect to age, pregnancy history, or symptoms at the time of initial evaluation. In addition, MRSA and MSSA patients did not differ in terms of potential risk factors for infection, such as diabetes, group B β-hemolytic streptococcus colonization, artificial rupture of membranes, epidural anesthesia, vaginal lacerations, episiotomy, cesarean section, or intrapartum antibiotic use (Table).

Ten (48%) MRSA and 11 (41%) MSSA patients required hospitalization. Although these inpatients did not

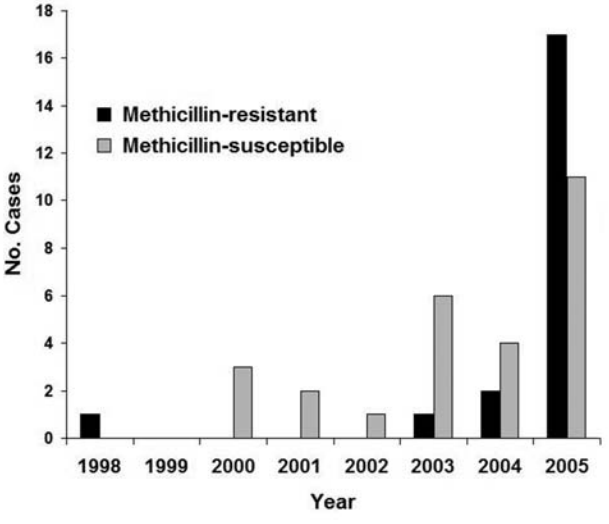

Figure 1. Cases of *Staphylococcus aureus*–associated postpartum mastitis at a single institution, 1998–2005. Cochrane-Armitage test for linear trend suggests a relative increase in methicillin-resistant cases during the study period; p = 0.04.

*Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA

Table. Demographics, symptoms, interventions, and outcomes in patients with *Staphylococcus aureus*–associated postpartum mastitis*

Variable	No. (%) patients		OR (95% CI)	p value*
	MRSA (n = 21)	MSSA (n = 27)		
Demographics				
Median age, y	32	32.5	–	0.90
Multiparous	12 (57)	9 (33)	2.67 (0.71–10.4)	0.10
Race†				
Caucasian	15 (71)	19 (79)	0.66 (0.13–3.19)	0.55
Other	6 (29)	5 (21)	1.52 (0.31–7.59)	0.55
Clinical symptoms				
Fever	7 (33)	10 (37)	0.85 (0.22–3.30)	0.79
Skin changes or fissures	6 (29)	8 (30)	0.95 (0.23–3.96)	0.94
Induration	20 (95)	21 (78)	5.71 (0.59–275.7)	0.09
Median time from delivery to symptom onset, d	27	33.5	–	0.60
Prenatal risk factors				
Diabetes	2 (10)	1 (4)	2.74 (0.13–167.56)	0.41
Group B β-hemolytic streptococcus colonization‡	3 (16)	4 (16)	0.88 (0.11–5.96)	0.87
Intrapartum risk factors				
Intrapartum treatment with antimicrobial drugs†	9 (43)	7 (29)	1.82 (0.45–7.52)	0.34
Cesarean section‡	3 (14)	2 (8)	1.92 (0.19–24.89)	0.50
Artificial rupture of membranes§	12 (63)	10 (44)	2.23 (0.54–9.41)	0.20
Vaginal laceration or episiotomy§	18 (95)	22 (96)	0.82 (0.01–67.75)	0.89
Epidural anesthesia§	12 (63)	18 (78)	0.48 (0.10–2.26)	0.28
Interventions				
Aspiration	17 (81)	22 (82)	0.97 (0.18–5.66)	0.96
Repeat aspiration	7 (41)	5 (23)	2.38 (0.48–12.14)	0.22
Incision and drainage	1 (6)	9 (41)	0.09 (0.00–0.84)	0.01
Drain placement	3 (14)	6 (22)	0.58 (0.08–3.26)	0.49
Outcomes				
Hospital admission	10	11	1.32 (0.36–4.90)	0.63
Median length of stay, d	4	4	–	0.90
Median leukocyte count, cells/μL	12.8	15.3	–	0.21
Temperature >38.1°C	6 (60)	2 (18)	6.75 (0.69–88.48)	0.05
Recurrent symptoms requiring readmission	1 (10)	1 (9)	1.11 (0.01–95.83)	0.94
Outpatient, later admitted	2 (18)	1(6)	3.75 (0.16–235.66)	0.29
Breastfeeding discontinued¶	3 (16)	5 (22)	0.71 (0.10–4.38)	0.67

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; OR, odds ratio for categorical variables using χ^2 analysis or Fisher exact test where appropriate; CI, confidence interval; p values for continuous variables calculated by using the Wilcoxon test.
†Data available for 25 MSSA patients.
‡Data available for 24 MSSA patients.
§Data available for 19 MRSA and 23 MSSA patients.
¶Data available for 19 MRSA and 24 MSSA patients.

differ in duration of symptoms before admission, length of stay, or leukocyte count, MRSA patients were more likely to have fever. One patient in each group required readmission for recurrent symptoms (Table).

Forty-six study patients had an abscess associated with mastitis; most (39 patients) underwent needle aspiration. Of these patients, 7 (41%) MRSA and 5 (23%) MSSA patients required repeat aspiration. Notably, 9 MSSA patients underwent incision and drainage a median of 4.5 days after aspiration (range 0–17 days), whereas only 1 MRSA patient required subsequent débridement (1 day later). Reasons for this difference are not clear; however, the more frequent use of serial ultrasound-guided aspiration in breast abscess management in recent years (when most MRSA cases occurred) may account for this finding.

In 17 of 21 MRSA cases, antibiotic use was documented. Twelve patients received antibiotics effective against MRSA, but only 2 received effective coverage at therapy onset (both received clindamycin). Patients initially received a penicillinase-resistant penicillin (10 patients), a first-generation cephalosporin (3 patients), a β-lactam/β-lactamase inhibitor (1 patient), or some combination of the above (6 patients). Median time to effective coverage for MRSA was 5 days (range 0–16 days); adequate antimicrobial agents included vancomycin (4 patients), trimethoprim-sulfamethoxazole (1 patient), clindamycin (9 patients), rifampin (2 patients), or some combination of the above (4 patients). Median duration of therapy, documented in 8 of 12 effective regimens, was 19 days (range 14–62 days).

Antimicrobial agent use was documented for 18 of 27 MSSA cases; in all 18 cases, isolates were susceptible to the initial antibiotic of choice. Initial regimens included penicillinase-resistant penicillins (10 patients), first-generation cephalosporins (2 patients), macrolides (1 patient), tetracyclines (1 patient), β-lactam/β-lactamase inhibitors (1 patient), vancomycin (1 patient), and clindamycin (2 patients). Duration of therapy for MSSA PPM, documented in 12 of 18 cases, was a median of 13.5 days (range 9–27 days).

Medical record review of affected patients did not show transmission of *S. aureus* to infants or other family members. In 1 MRSA patient, a perirectal abscess developed 5 months after the mastitis resolved. Intraoperative cultures of the abscess grew MRSA with identical susceptibilities, which suggests persistent colonization; however, typing of the isolates was not performed.

Of 21 MRSA isolates available for PCR analysis, 20 possessed SCC*mec* IV. The remaining isolate contained SCC*mec* II (Figure 2) and displayed resistance to clindamycin. In contrast, 95% of isolates with SCC *mec* IV were clindamycin susceptible.

Conclusions

To our knowledge, this is the largest case-control study of patients with MRSA-associated PPM. Although *S. aureus* is the most common etiologic agent of PPM, cases caused by MRSA have rarely been described. Epidemic MRSA cases, linked to the hospital transmission of a community-acquired isolate, have been observed more recently (*6*). Our study suggests that CA-MRSA is an increasingly common pathogen in spontaneous cases of PPM.

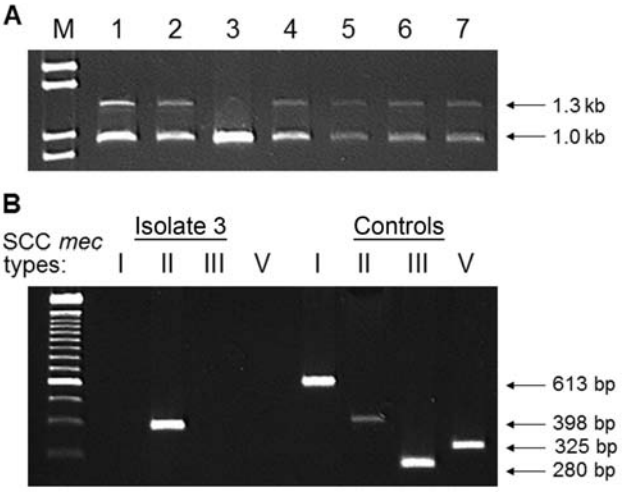

Figure 2. A) PCR with specific primers for class B *mec* complex (1.3 kb) and type 2 *ccr* complex (1.0 kb) identifies isolates containing *Staphylococcus* cassette chromosome (SCC) *mec* type IV: lanes 1, 2, and 4-7. B) When control strains are used, PCR identifies SCC*mec* type II in isolate 3. Lane M, molecular mass marker

PPM due to CA-MRSA appears to be increasing at our institution. Among 17 MRSA-infected mothers in 2005, delivery dates spanned >9 months without overlap, which suggests that MRSA was independently acquired rather than outbreak-related. In addition, although isolates were not subjected to molecular typing by pulsed-field gel electrophoresis, PCR results suggest that 16 (94%) of MRSA isolates in 2005 were community-acquired.

The epidemiology of CA-MRSA PPM is poorly understood. Notably, nearly twice as many MRSA-infected than MSSA-infected women were multiparous in this study (57% vs. 33%, respectively). The prevalence of CA-MRSA is increasing among young children, and intrafamilial transmission of isolates has been documented (*11*,*12*). Therefore, mothers with young children may be at increased risk for CA-MRSA PPM. Alternatively, these patients may serve as a reservoir for MRSA in the community, transmitting this organism to family members.

In the current study, women with MRSA were significantly less likely to receive adequate and timely antimicrobial drug treatment, but consequences of this difference are unclear. Lee et al. suggest that small CA-MRSA abscesses in children can be managed effectively with incision and drainage alone (*13*). Indeed, most women in this study underwent incision and drainage or wound aspiration without significant differences in outcomes. Although MSSA patients were more likely to undergo breast abscess incision and drainage than their MRSA counterparts, both methods are considered appropriate surgical interventions (*14*).

Although related cases of infant infection were not found, charts of household contacts were not reviewed in this study; cases of *S. aureus* transmission to infants or other family members may have been undetected. Several authors have reported mother-to-infant transmission of MRSA through breast milk (*15*,*16*). Although decolonization measures in MRSA-colonized patients have not demonstrated long-term effectiveness (*17*), the possibility of infant MRSA acquisition may warrant further evaluation of such measures in infected, breastfeeding mothers.

As with any retrospective case-control study, ours had several limitations. First, the study population is small, which limits the generalizability of the results. Second, patients were added to the study by using results of positive cultures; consequently, cases likely represented more severe and complicated infections in which cultures were necessary after routine therapeutic measures failed. Third, although PPM has been associated with multiple patient factors (i.e., difficulty breastfeeding, tobacco use, and stress), a thorough risk assessment is limited by retrospective study. In addition, medical record review may not indicate certain CA-MRSA risk factors, such as socioeconomic status, history of incarceration, or exposure to day care facilities. Finally, although the study results suggest a

recent increase in MRSA PPM, an assessment of incidence would require further prospective analysis.

In summary, CA-MRSA has emerged as an increasingly common pathogen in PPM. Therapy against CA-MRSA should be considered in refractory or severe cases of PPM until wound, drainage, or breast milk cultures can be obtained. Adjunct surgical drainage or aspiration is often warranted in such cases. Additional study is required to determine the utility of routine cultures in postpartum mastitis, the prevalence of CA-MRSA in this emerging problem, and the consequences of CA-MRSA colonization for breastfeeding infants.

Acknowledgments

MSSA and MRSA isolates were identified from the microbiology database by Mike Malczynski. MRSA strains used as controls for SCC*mec* typing, including type I (NCTC10442), type II (N315), type III (85/2082), type IV (CA05), and type V (WIS [WBG8318]-JCSC3624), were kindly provided by Dr Teruyo Ito. We also thank Dr Patricia Garcia for her support and guidance.

Dr Reddy is currently a fellow in the Division of Infectious Diseases at Northwestern University Feinberg School of Medicine. Her research and clinical interests include surgery-related infections and multidrug-resistant, nosocomial pathogens.

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Address for correspondence: Pavani Reddy, Division of Infectious Diseases, Northwestern University Feinberg School of Medicine, 676 N Saint Clair St, Suite 200, Chicago, IL 60611, USA; email: p-reddy2@md.northwestern.edu

Disseminated Neonatal Herpes Caused by Herpes Simplex Virus Types 1 and 2

Aleksandra Knezevic,* Jelena Martic,† Maja Stanojevic,* Sasa Jankovic,‡ Jasminka Nedeljkovic,‡ Ljubica Nikolic,† Srdjan Pasic,† Borisav Jankovic,† and Tanja Jovanovic*

Disseminated neonatal herpes simplex virus (HSV) infection is characterized by progressive multiple organ failure and high mortality rates. It can result from infection with either HSV-1 or HSV-2. We report a case of disseminated neonatal herpes that was caused by HSV-1 and HSV-2.

Neonatal herpes simplex virus (HSV) infection is among the most severe perinatal infections. Most (85%) neonatal HSV infections are acquired during delivery, although in utero (5%) and postnatal (10%) infections do occur (*1*). The risk for transmission to the newborn is much higher in women with primary HSV infections (*2*). Neonatal herpes can be localized to skin, eyes, and mouth (≈45% of cases), involve the central nervous system (≈30% of cases), or can cause disseminated infection involving multiple organs such as liver, lungs, adrenal glands, and brain (≈25% of cases).

Disseminated infection is the most severe form of neonatal herpes, with a mortality rate of 85% for untreated neonates (*3*). It is usually observed when the infant is 5–9 days old; signs include irritability, seizures, respiratory distress, jaundice, bleeding diatheses, shock, and often vesicular exanthema (*3*,*4*). Early treatment with high-dose acyclovir reduces the mortality rate (*5*). Early recognition of disseminated infection is difficult because of nonspecific symptoms and signs of sepsis and because initiation of antiviral therapy is often delayed (*1*). The high risk for death requires prompt diagnostic evaluation that includes testing by HSV DNA PCR as the preferred method or virus culture (*6*,*7*).

Neonatal herpes can result from infection with either HSV-1 or HSV-2; the latter is associated with a poorer prognosis (*7*). We report a case of disseminated neonatal herpes infection with HSV-1 and HSV-2.

The Case

A full-term infant girl had febrile illness and lethargy and fed poorly at 3 days of age. She was born by normal vaginal delivery with prolonged expulsion of placental membranes. Initial laboratory investigations showed a normal leukocyte count (11,100 cells/μL), a slightly elevated C-reactive protein level (18 mg/L), and elevated levels of liver enzymes (aspartate aminotransferase [AST] 283 U/L and alanine aminotransferase [ALT] 111 U/L). A screen for sepsis was performed and she was treated with broad-spectrum antimicrobial drugs. On day 4 of life, signs of respiratory distress appeared and intermittent mechanical ventilation was initiated. A chest radiograph showed streaky and patchy bilateral pulmonary opacities and right-side pleural effusion. Neurologic examination showed mild hypotonia. On day 6 of life, she was still febrile with thrombocytopenia (56,000 cells/μL), an increased C-reactive protein level (32 mg/L), and signs of fulminate liver failure (AST 13,740 U/L and ALT 3,180 U/L) and marked coagulopathy. Despite full intensive care support, she died of rapidly progressive multiple organ failure on day 9.

Postmortem findings showed widespread necrosis of lungs, liver, and adrenal glands. Serologic investigations showed no immunoglobulin M (IgM) and IgG antibodies for HSV-1 and HSV-2. An ELISA (Virion/Serion, Würzburg, Germany) detected IgG antibodies for rubella virus, cytomegalovirus, varicella zoster virus, parainfluenza virus, adenovirus, and coxsackie B virus; however, these results were not indicative of active infection. Blood and urine bacterial cultures were negative. Retrospective virologic examination of postmortem specimens (tracheal aspirate, liver, lungs, and stomach) in different cell lines (Vero, RD, L20B) showed cytopathogenic virus, which was suggestive of HSV that was identified by PCR.

Viral DNA was extracted from all postmortem specimens (tracheal aspirate, liver, lungs, and gut) and all virus-positive cultures of tracheal aspirate, liver, lungs, and stomach in different cell cultures (Vero, RD, L20B) by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). The DNA was then used for HSV DNA PCR. Target DNA was amplified with primers for the HSV-1 thymidine kinase gene (Fw 5′-AGCGTCTTGTCATTGGCGAA-3′ and Rev 5′-TTTTCTGCTCCAGGCGGACT-3′) and for the HSV-2 DNA polymerase gene (Fw 5′-CGTCCTG GAGTTTGACAGCG-3′ and Rev 5′-CAGCAGC GAGTCCTGCACACAA-3′) (*8*). A 342-bp band for HSV-1 and a 445-bp band for HSV-2 were found in all postmortem specimens (Figure) and in all virus-positive cultures examined.

*University of Belgrade School of Medicine, Belgrade, Serbia; †Mother and Child Health Institute Dr Vukan Cupic, Belgrade, Serbia; and ‡Institute of Immunology and Virology Torlak, Belgrade, Serbia

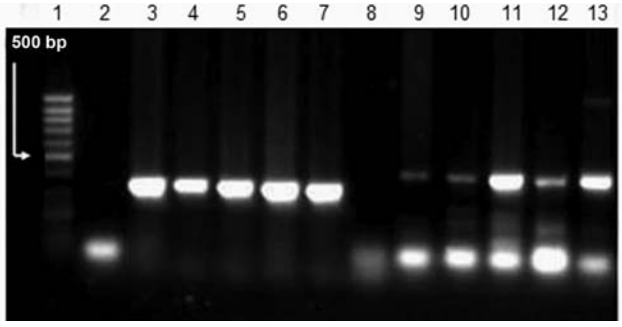

Figure. Results of herpes simplex virus (HSV) type 1 and type 2 PCRs in postmortem specimens. Lane 1, 100-bp DNA ladder; lane 2, negative control HSV-1; lane 3, positive control HSV-1; lane 4, tracheal aspirate HSV-1; lane 5, liver HSV-1; lane 6, lung HSV-1; lane 7, stomach HSV-1; lane 8, negative control HSV-2; lane 9, positive control HSV-2; lane 10, tracheal aspirate HSV-2; lane 11, liver HSV-2; lane 12, lung HSV-2; lane 13, stomach HSV-2.

Nucleotide sequence analysis was performed by using the ABI Prism BigDye 3.1 sequencing system (Applied Biosystems, Foster City, CA, USA) and showed identical sequences in different specimens. When these sequences were compared with those available in the GenBank database by using the BLAST tool (www.ncbi.nlm.nih.gov/BLAST/), the highest similarity was observed for relevant HSV genes, namely HSV-1 strain CL 101 and HSV-2 strain KN 53690.

Retrospective serologic examination of maternal samples and avidity tests at 3 different time points detected IgM and IgG antibodies initially for HSV-1 and subsequently for HSV-2 (Table); the increase in IgG avidity to both virus types correlated with primary infection.

Conclusions

Neonatal disseminated HSV infection is most frequently caused by HSV-2, although HSV-1 can also be the cause. To the best of our knowledge, our patient is the first PCR-confirmed case of disseminated neonatal herpes caused by concomitant infection with HSV-1 and HSV-2.

Prompt diagnosis was difficult because of the early appearance of nonspecific symptoms (day 3), signs of respiratory distress (day 4), and rapid development of multiple organ failure (day 6). Oral and skin vesicular lesions were not detected, and the mother had no history of herpes infection. Serologic HSV status of the newborn was not of great clinical value. Postmortem virologic examination including viral isolation and HSV DNA PCR identified HSV-1 and HSV-2.

The results of retrospective serologic examination for maternal IgM and IgG antibodies to HSV-1 and HSV-2 and avidity tests suggested that primary maternal HSV infection occurred near the time of delivery. Because of the 2-month delay in obtaining maternal serologic results, whether the mother was infected by both HSV types concomitantly or successively near the time of delivery is unclear. These results suggest that the newborn acquired the infection during delivery, although in utero infection cannot be ruled out. The rapid onset of disseminated neonatal HSV infection (day 3) and development of multiple organ failure seen in this patient may be the result of concomitant infection with HSV-1 and HSV-2.

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Dr Knezevic is a medical microbiologist and assistant professor of microbiology and immunology at the Institute of Microbiology and Immunology, School of Medicine, University of Belgrade. Her research interests include persistent viral infections, in particular herpesvirus and human papillomavirus infections.

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Table. Maternal serologic status for herpes simplex virus (HSV)–1 and HSV-2 at different time points after delivery*

	HSV-1†		HSV-2†	
Time after delivery, mo	IgM (IU/mL)	IgG (IU/mL)	IgM (IU/mL)	IgG (IU/mL)
2	170	836 Avidity 57%	35	–
6	145	1,019 Avidity 90%	50	135 Avidity 50%
9	57	550	—	55 Avidity 77%

*IgM, immunoglobulin M.
†Reference values: IgM positive >30 IU/mL; IgG positive >30 IU/mL.

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Address for correspondence: Aleksandra Knezevic, Institute of Microbiology and Immunology, School of Medicine, University of Belgrade, Dr Subotica 1/1, 11000 Belgrade, Serbia; email: aknezevic@med.bg.ac.yu

Surveillance for West Nile Virus in Clinic-admitted Raptors, Colorado

Nicole Nemeth,*† Gail Kratz,*‡ Eric Edwards,† Judy Scherpelz,*‡ Richard Bowen,* and Nicholas Komar†

In 2005, 13.5% of clinic-admitted raptors in northern Colorado tested positive for West Nile virus (WNV). Clinic-admitted–raptor surveillance detected WNV activity nearly 14 weeks earlier than other surveillance systems. WNV surveillance using live raptor admissions to rehabilitation clinics may offer a novel surveillance method and should be considered along with other techniques already in use.

West Nile virus (WNV; genus *Flavivirus*; family *Flaviviridae*) is an emerging pathogen of public health and veterinary importance. In North America, WNV has been associated with death in >198 species of birds, including ≥33 species of raptors (*1*). Many hawk and owl species are known to survive WNV infection (*2–5*). Presumably most raptors become infected from mosquito bites; however, some evidence suggests that infection may occur after consumption of infected prey items (*2*,*4–6*). Thus, raptors may be infected at a greater rate than nonraptors. Dead raptors and other birds (particularly corvids) have been used for early detection of WNV activity (*7*). However, once WNV activity is established in a location, birds that are highly susceptible to fatal infection are removed from the environment, and as a result, avian death rates should diminish (*8*). Raptors infected with WNV that are admitted to rehabilitation facilities, either because of WNV-associated illness or injury or for other unrelated complications, may serve as an alternate source for early detection of WNV infection.

The Study

From 2002 through 2005, raptors originating in Colorado were bled by ulnar venipuncture and orally swabbed upon admission to the Rocky Mountain Raptor Program of Colorado State University. WNV was first detected in Colorado in August 2002, and testing of raptors was initiated in September (oral swabs) and October (serum samples). In all other years, samples were collected from early to late April through mid to late October. Specimens were tested for WNV-neutralizing antibodies by plaque-reduction neutralization test (PRNT) and for virus isolation by Vero cell plaque assay (*9*) or WNV antigen by VecTest WNV Antigen Detection Assay (Medical Analysis Systems, Ventura, CA, USA). Isolated viruses were identified as WNV by VecTest. To confirm that antibody-positive adult raptors were recently infected, we evaluated 90% neutralization titers in acute-phase and convalescent-phase serum samples collected ≈3 weeks apart. A 4-fold increase in titer was considered evidence of a recent infection. Cross-reactivity for another closely related North American flavivirus, Saint Louis encephalitis virus, was ruled out by comparing 90% neutralization titers. A 4-fold greater titer for 1 of the viruses indicated that particular virus as the etiologic agent for the infection. Utility of WNV detection in raptors was evaluated in relation to other existing WNV surveillance techniques in northern Colorado.

We report results from 323 raptors sampled from 2002 through 2005. Most of these (83%) originated from Weld and Larimer counties, which represent an area of 6,639 square miles, larger than Connecticut and Rhode Island combined. During the study, 38 raptors (11.8%) tested positive for WNV. Some were positive by both oral swab and seroconversion, while others were positive according to only 1 of these. Usually, birds that were positive only by oral swab died before 1 or both blood samples could be collected, so we were unable to test for seroconversion.

In 2002, 17 raptors were tested (blood by PRNT and oral swab by plaque assay), 4 of which were seropositive for WNV between October 7 and November 15. In 2003, 52 birds were tested (serum by PRNT and oral swab by VecTest), 7 of which seroconverted and 5 of which were oral swab–positive. Positive samples were detected between July 17 and September 1. In 2004, 113 birds were tested by plaque assay of oral swab (no blood test), and 3 were found to be positive between July 28 and September 17. In 2005, 141 birds were tested (serum by PRNT and oral swab by plaque assay), of which 19 were positive (8 by seroconversion, 6 by virus isolation from swab, and 5 by both methods; Table). Positive results were from birds admitted between April 8 and September 21.

To compare our test results with those from other surveillance systems for WNV, we limited our data to specimens collected April 1–October 15, 2005, from raptors originating in Weld or Larimer counties. In comparing the earliest date of detection for each of the surveillance methods in place in these counties, clinic-admitted raptor surveillance provided the earliest evidence of WNV activity (April 8), preceding all other WNV surveillance systems'

*Colorado State University, Fort Collins, Colorado, USA; †Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; and ‡Rocky Mountain Raptor Program, Fort Collins, Colorado, USA

Table. Results of West Nile virus testing in Colorado raptors admitted to a rehabilitation clinic, April 1–October 15, 2005

Species	No. tested	No. positive (%)	No. seroconverted	No. swab positive	Earliest date of detection
Swainson's hawk	28	8 (28.6)	5	3	Jun 27
Red-tailed hawk	13	4 (30.8)	2	3	Jul 28
Ferruginous hawk	1	1 (100.0)	0	1	Jul 31
American kestrel*	32	0	–	–	–
Peregrine falcon	2	1 (50.0)	1	1	Aug 16
Golden eagle	3	1 (33.3)	1	1	Aug 18
Great horned owl	23	4 (17.4)	3	2	Apr 8
Barn owl	24	0	–	–	–
Long-eared owl	5	0	–	–	–
Other species†	10	0	–	–	–
Total	141	19 (13.5)	12	11	Apr 8

*The proportion of positives for kestrel was significantly less than for all other species combined ($p = 0.0046$, $\alpha = 0.0056$; Fisher exact test with Bonferroni adjustment for 9 comparisons). No other statistically significant associations were observed for the species tested.
†Includes burrowing owl (n = 2), sharp-shinned hawk (n = 2), Cooper's hawk (n = 1), eastern screech owl (n = 1), merlin (n = 1), osprey (n = 1), prairie falcon (n = 1), and northern saw-whet owl (n = 1).

initial detections of WNV activity by nearly 14 weeks (Figure).

Conclusions

The early detection of WNV in clinic-admitted raptors compared with other detections by surveillance systems in northern Colorado during 2005 points to the potential utility of raptor rehabilitation centers for WNV surveillance. Although other active surveillance systems require significant allocations of human resources, clinic-admitted raptor surveillance is a passive system that takes advantage of existing resources outside the traditional public health infrastructure. Nationwide, about 1,000 wildlife rehabilitation facilities admit ≈10,000 birds annually (P. Redig, pers. comm.). Participation in surveillance efforts provides rehabilitators with valuable diagnostic information and can be accomplished at no cost to the rehabilitator, provided that provisions are supplied.

The detection of WNV in an oral swab of a great horned owl in early April in Colorado was quite unexpected because of the early date. This bird was an uninjured nestling that was brought to the clinic for nurturing until it could be replaced into its original nest. The oral swab yielded a low number of infectious virus particles (2.5 PFU), and the nestling failed to develop clinical signs and failed to seroconvert. We believe that the oral cavity may have been contaminated by a recent prey meal provided by the bird's parents shortly before admission. Although early spring transmission of WNV by mosquitoes to either the owlet or a prey animal is possible, persistent infection of the prey item is an alternative explanation. Experimentally infected hamsters develop chronically infected kidneys (*10*), and birds may also maintain persistent visceral infections (*2*).

If the early detection in the owl was an anomaly, the next earliest evidence of WNV activity from clinic-admitted raptors was June 28, which also preceded all other detections. The first confirmed human case of West Nile fever in the study area developed symptoms on July 17, and the first confirmed case of West Nile neurologic disease occurred on August 6 (Figure).

Although we have shown that a combination of serologic and oral swab testing increases the sensitivity of clinic-admitted raptor surveillance almost 2-fold, serologic testing has 3 important limitations: 1) blood sampling requires special training and expertise; 2) evidence of seroconversion requires 2 samples spaced apart by at least 2 weeks, and therefore reporting of positive results is significantly delayed by several weeks after onset of infection; and 3) neutralization tests can be prohibitively expensive and require extensive training, time, supplies, Biosafety Level-3 (BSL-3) lab facilities, and expertise in interpreting results, which are complicated by cross-reactions with closely related viruses. Limiting sampling to oral swabs reduces sensitivity; however, the savings in time and cost

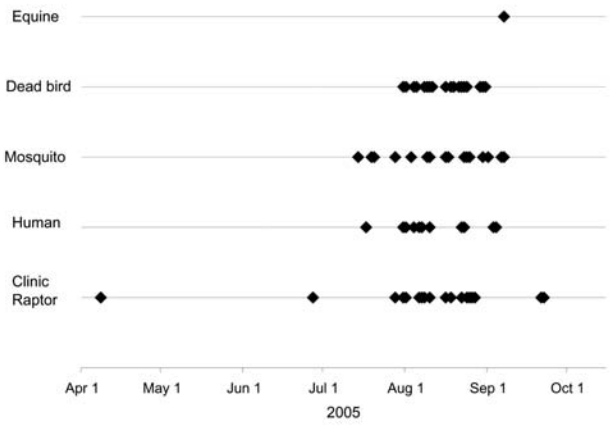

Figure. Chronology of detections of West Nile virus by various surveillance systems in place in Larimer and Weld Counties, northern Colorado, 2005. Confirmed human cases, mosquito, dead bird, and equine surveillance information provided by the Centers for Disease Control and Prevention's ArboNet Surveillance System through October 15, 2005.

would permit a greater number of samples to be collected and tested. Although we used plaque assay for detecting WNV in oral swabs, which also requires BSL-3 laboratory facilities, our samples could have been tested with high sensitivity and specificity for WNV-specific RNA sequences by using reverse transcription–PCR, which requires a lower level of biosafety (*11*).

In conclusion, limited data from 1 small region of North America suggest that WNV surveillance using live raptor admissions to rehabilitation facilities should be considered along with other established surveillance methods already in use (*12*,*13*). Clinic-admitted raptors are most useful for early detection or continued detection of WNV activity. However, this form of surveillance is inadequate for quantifying local transmission risk.

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Dr Nemeth is a graduate student at Colorado State University and worked for several years in the Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA. Her research interests include avian infectious diseases and the conservation and ecology of birds and other wildlife.

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Address for correspondence: Nicole Nemeth, 3801 W Rampart Rd, Colorado State University, Fort Collins, CO 80523-1683, USA; email: nnemeth@colostate.edu

Mosquitoborne Infections after Hurricane Jeanne, Haiti, 2004

Mark E. Beatty,* Elizabeth Hunsperger,* Earl Long,† Julia Schürch,‡ Seema Jain,† Rom Colindres,† Gerald Lerebours,§ Yves-Marie Bernard,¶ James Goodman Dobbins,# Mathew Brown,¶ and Gary G. Clark,*,**

After Hurricane Jeanne in September 2004, surveillance for mosquitoborne diseases in Gonaïves, Haiti, identified 3 patients with malaria, 2 with acute dengue infections, and 2 with acute West Nile virus infections among 116 febrile patients. These are the first reported human West Nile virus infections on the island of Hispaniola.

Hurricane Jeanne caused large-scale devastation in Gonaïves, Haiti, on September 18, 2004. The US Department of Health and Human Services assisted the Haitian Ministry of Health by conducting a rapid field assessment of health-related issues. Among the actions recommended by the team was immediate epidemiologic assistance from the Centers for Disease Control and Prevention (CDC) to reinforce and expand epidemiologic surveillance to identify as early as possible any emerging epidemic or community health problems. Concern was raised that the combination of flooding, loss of shelter, and destruction of infrastructure would result in an outbreak of mosquitoborne diseases. We conducted surveillance to assess the extent of mosquitoborne diseases and monitor for outbreaks of these diseases.

The Study

We established laboratory-based fever surveillance at the 3 clinics providing healthcare in Gonaïves after the passage of Hurricane Jeanne. Febrile patients (core temperature ≥38.5°C when first assessed) were asked to provide blood for a serum sample and thick and thin malaria smears. The attending physician recorded each patient's medical history, conducted a physical examination, and reported the discharge diagnosis and the therapy that was provided. We asked patients to return in 2 weeks so that a convalescent-phase serum sample could be collected.

Malaria smears were stained and read by using standard methods (*1*) at CDC. To diagnose dengue, we used nested PCR and the TaqMan assay to detect dengue viral RNA in serum samples obtained ≤5 days after onset of symptoms (*2*,*3*). In addition, we used an immunoglobulin M (IgM) antibody-capture (MAC)–ELISA to detect anti-dengue IgM antibodies in all serum specimens (*4*) at CDC. A result was considered positive when optical density, after comparison to negative serum and control antigen, was >0.20. All serum specimens were also tested for the presence of IgG antibodies to determine previous exposure to flaviviruses by using an IgG ELISA. In paired samples, a full titration of 4-fold dilutions of serum was used. The endpoint titration of IgG was determined to assess seroconversion (*5*). Each plate was compared with a negative control serum specimen. Because of cross-reactivity between anti-flavivirus antibodies, we used a microsphere-based immunoassay (MIA) with a quadratic discrimination analysis (*6*) and a plaque reduction neutralization test (PRNT) to distinguish between infecting flaviviruses. For the PRNT, serial dilutions of heat-inactivated serum were incubated with defined amounts of West Nile, Saint Louis encephalitis, and dengue viruses 1–4 for 2 hours at room temperature. The nonneutralized viral fraction was subsequently adsorbed onto a monolayer of Vero cells for 1 hour. The resultant plaques were counted and compared with results for the control virus with no serum. The endpoint of the titration was the highest dilution of serum that reduced the number of plaques 90% compared with the control results.

From November 15 through December 22, 2004, 116 acutely febrile patients were identified and included in our surveillance. Ages ranged from 4 months to 71 years (median 4 years); 52% were female. All patients lived in Gonaïves. Seventy-one patients (61%) appeared for treatment with a chief complaint of fever with cough, 35 (30%) had fever with no apparent source, 6 (5%) reported fever with diarrhea, and 4 (3%) reported fever with rash. Patients sought treatment a median of 3 days after the onset of fever (range 0–28 days). In addition to fever, the most commonly reported symptoms were cough (77, 66%), abdominal pain (57, 49%), and headache (56, 48%). Thirty-nine patients (34%) had at least 1 clinical sign of dehydration; 16 patients (14%) were hypotensive on physical examination. No patients were jaundiced or had spontaneous hemorrhage. The most common clinical diagnoses were upper respiratory infection (35, 30%), malaria (34, 29%), pneumonia (21, 18%), and typhoid fever (13, 11%). No cases of

*Centers for Disease Control and Prevention, San Juan, Puerto Rico, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA ; ‡Médecins Sans Frontières, Belgium; §John Snow Incorporated, Port-au-Prince, Haiti; ¶Centers for Disease Control and Prevention, Port-au-Prince, Haiti; #Pan American Health Organization, Port-au-Prince, Haiti; and **Agricultural Research Service, Gainesville, Florida, USA

dengue fever were suspected. Fifty-eight patients (50%) were treated with oral antimicrobial drugs; 13 patients (11%) were prescribed chloroquine, and 2 patients (2%) received an antihelminthic drug. All cases of suspected malaria were diagnosed by patients' clinical symptoms. Suspected tuberculosis was confirmed in 1 patient by a positive sputum smear. None of 116 patients was admitted to a hospital.

Of the 116 thick and thin smears, 3 (3%) samples showed a high level of parasitemia with *Plasmodium falciparum*. The 3 corresponding patients had fever with no apparent source. Malaria was suspected in 2 of the patients by their clinical symptoms; the third patient was thought to have typhoid and was treated with trimethoprim-sulfamethoxazole.

Two patients (2%) had acute, secondary dengue infections that were confirmed as positive by both IgM and IgG serologic tests. Both patients had a chief report of fever with no source, but malaria was suspected by the attending physician, and 1 patient was treated with chloroquine. We were not able identify dengue viral particles in the serum specimens of these patients. However, 79 patients (68%) were positive for anti-dengue IgG, which suggests a high level of flavivirus transmission in this area in the recent past (Figure).

Two patients (2%) had MIA results consistent with acute West Nile virus infection. The results were confirmed by PRNT (Table). Both patients were febrile in the clinic; 1 was a 13-year-old boy and the other was an infant girl <1 year of age. In addition to fever, the 13-year-old reported headache and abdominal pain, while cough was reported in the infant. Acute malaria was clinically diagnosed in both patients. The older child received chloroquine, while the younger child received only acetaminophen for fever control.

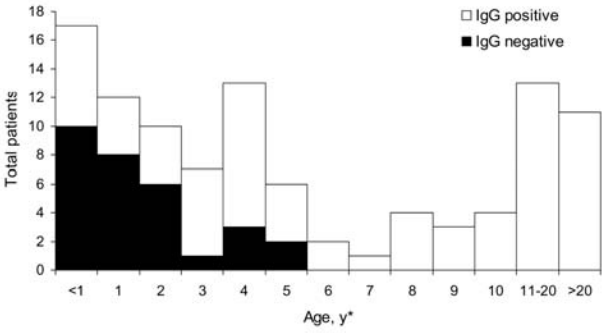

Figure. Results of immunoglobulin G (IgG) ELISA for antiflavivirus antibodies among patients exhibiting fever, Gonaïves, Haiti, October 2004 (n = 105). *Exact ages are not available for 11 patients.

Conclusions

This surveillance program was established to assess the incidence of vectorborne diseases in the wake of Hurricane Jeanne. A total of 116 acutely febrile patients had blood drawn to determine whether a mosquitoborne disease was the etiologic agent of fever. An outbreak of mosquitoborne disease was not detected during the period of surveillance. Our data are consistent with previously published reports, which indicate that the incidence of arboviral infections rarely increases after water-related disasters (e.g., floods, hurricanes) (*7*–*9*). However, malaria outbreaks are common in such settings (*9*,*10*).

Despite the absence of an outbreak, our surveillance did identify the ongoing transmission of 3 mosquitoborne pathogens. Specifically, we diagnosed 3 cases of acute malaria, 2 cases of acute dengue, and 2 cases of acute West Nile virus infection. We also detected a high seroprevalence of dengue infections in children, which suggests substantial local dengue transmission in the Gonaïves area in the recent past.

The high seroprevalence of dengue and the low smear-positive rate of malaria from our surveillance were consistent with previously reported studies in this region of Haiti (*11*,*12*). The identification of 2 patients with positive West Nile virus results in Haiti is new. The only other human West Nile virus infections identified in the Caribbean Basin were 1 case reported in a Cayman Islands resident in 2001 (*13*) and 2 cases reported in Cuba, 1 in 2003 and the other in 2004 (*14*). This finding is not unexpected, however, because Komar et al. have identified West Nile virus in bird species native to the Dominican Republic (*15*), located to the east of Haiti on the island of Hispaniola.

The fact that the rate of West Nile virus infection was equal to the rate of acute dengue infection among our participants is of concern. Moreover, because both viruses can cause a nonlocalizing fever, the potential for confusion with malaria exists. Differentiating the cause of acute nonlocalizing febrile illnesses by examining malaria smears before initiating therapy, especially in an area with a history of low smear positivity, is therefore important.

Table. Endpoints for 90% plaque reduction neutralization tests for patients with acute West Nile virus (WNV) infection, Gonaïves, Haiti, 2004*

Age	Sex	DEN-1	DEN-2	DEN-3	DEN-4	SLE	WNV
13 y	Male	<100	<100	<100	<100	<100	200
<1 y	Female	<100	<100	<100	<100	<100	800

*DEN, dengue; SLE, Saint Louis encephalitis virus.

Acknowledgments

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Dr Beatty was previously the epidemiology and prevention activity leader at the Dengue Branch of CDC in San Juan, Puerto Rico. He joined the International Vaccine Institute in Seoul, Korea, in 2006. Dr Beatty's research interests include arboviral and enteric infectious diseases.

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Address for correspondence: Mark E. Beatty, Pediatric Dengue Vaccine Initiative, International Vaccine Institute, SNU Research Park, San 4-8 Bongcheon-7-dong, Kwanak-gu, Seoul, Republic of Korea 151-919; email: mbeatty@pdvi.org

Characteristics of *Staphylococcus aureus* Infections, Chicago Pediatric Hospital

Preeti Jaggi,* Suzanne M. Paule,† Lance R. Peterson,† and Tina Q. Tan*

Invasive and skin community-associated (CA)–methicillin-resistant *Staphylococcus aureus* isolates from children were matched with invasive CA–methicillin-sensitive *S. aureus* strains during 2000–2004. Isolates were analyzed for presence of Panton-Valentine leukocidin. A USA400 lineage clone (n = 6) and the predominant USA300 lineage clone emerged.

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections have been increasing in children since the 1990s. Panton-Valentine leukocidin (PVL) has been associated with CA-MRSA strains (*1–4*).

As CA-MRSA infections have been increasing in previously healthy pediatric patients, we sought to do the following: 1) describe the clonal relatedness of these CA-MRSA isolates by using pulsed-field gel electrophoresis (PFGE), 2) detect the presence of PVL genes among CA-MRSA pediatric isolates causing invasive disease and among isolates causing skin and soft tissue infections (SSTI), 3) determine clinical and epidemiologic differences among patients with invasive disease caused by community-associated methicillin-sensitive *S. aureus* (CA-MSSA) versus those with disease caused by CA-MRSA strains, 4) assess the geographic pattern of infection, and 5) measure the antimicrobial agent susceptibility for CA-MRSA strains.

The institutional review board at Children's Memorial Hospital, a 253-bed, freestanding children's hospital in Chicago, Illinois, approved this study. A CA-MRSA strain was defined as a clinical MRSA isolate recovered from a pediatric patient (<1–18 years of age) who had no established risk factors for MRSA infection (no residence in long-term care facility, no hospitalization except for routine birth, and no permanent indwelling medical devices). For most patients, strains were recovered within 72 hours of admission. Exceptions included patients who had clinical evidence of community-associated disease and whose isolates were obtained after 72 hours of hospitalization. Isolates recovered from normally sterile sites were defined as invasive. We identified patients with *S. aureus* infections retrospectively by reviewing microbiology log books from March 1, 2000, through November 30, 2004.

Demographic and clinical data retrieved included age, sex, race, ZIP code of residence, length of hospitalization, and clinical outcomes. When possible, patients with invasive cases were matched to patients with CA-MRSA SSTI and to those with invasive CA-MSSA infections by age (within 12 months for those <18 months or within 3 years for those >18 months), geographic location of patient residence, or year of infection.

S. aureus isolates were identified by standard microbiologic methods. For all *S. aureus* isolates that appeared erythromycin resistant and clindamycin susceptible, antibiotic double disk diffusion assay was performed (*5*).

Isolates of *S. aureus*, including control strain NCTC 8325 (Bio-Rad, Hercules, CA, USA), were analyzed by PFGE after DNA digestion with *Sma*I. Resulting fragments were separated by using the *Staphylococcus* program 5 (GenePath System, Bio-Rad), and DNA banding patterns were compared (*6,7*). PCR was used to detect the PVL genes (*8*).

Categorical variables were analyzed by χ^2 analysis. Variables with non-normal distribution were analyzed by Mann-Whitney U test; 2-tailed p value ≤ 0.05 was statistically significant (SPSS Inc., version 11.0, Chicago, IL, USA).

From 166 MRSA patient strains noted in the microbiology records, 21 patients with invasive CA-MRSA infection were identified. Three patient isolates were unavailable and were excluded from analysis. Patients with invasive CA-MRSA strains were case-matched with patients with CA-MRSA SSTI (16/18 were matched by age). During the study period, ≈31 cases of invasive CA-MSSA disease were identified and 10 cases were able to be retrieved and matched with cases of invasive CA-MRSA (9/10 case-patients were matched by age).

Groups with invasive CA-MRSA and groups with invasive CA-MSSA did not differ significantly regarding sex, initial leukocyte count, duration of fever, or length of hospital stay. Pediatric patients with invasive CA-MRSA infection were more likely to be African American (p = 0.01) and were febrile significantly longer than patients with invasive CA-MSSA (p = 0.03). One of the patients with invasive CA-MRSA died (Table 1).

Among the 18 patients with invasive CA-MRSA, 17 patients required surgical drainage, and 1 patient was given extracorporeal membranous oxygenation. Among the 10 patients with invasive CA-MSSA disease, all required surgical intervention, but none died. Of 18

*Northwestern University, Chicago, Illinois, USA; and †Evanston Northwestern Healthcare, Evanston, Illinois, USA

Table 1. Comparison of clinical characteristics between pediatric patients with invasive CA-MRSA and CA-MSSA*

Characteristic	Invasive CA-MRSA, n (%)	Invasive CA-MSSA, n (%)	p value
Race			
Black	16/18 (88)	3/10 (30)	0.01
Caucasian	0	2/10 (20)	0.01
Hispanic	1/18 (6)	5/10 (50)	0.01
Other	0	0	0.01
Unknown	1/18 (6)	1/10 (10)	0.01
Days of discordant therapy (CA-MRSA)			
Mean ± SD (range)	2.22 ± 1.76 (0–6)	N/A	
Days febrile, mean ± SD (range)	7.0 ± 4 (0–17)	5.20 ± 10.0 (0–32)	0.03
Days in hospital, mean ± SD (range)	14.2 ± 7.6 (4–28)	13.8 ± 16.8 (4–60)	NS
Diagnosis†			
Osteomyelitis, acute	3/18 (38)	1/10 (10)	NS
Ostyeomyelitis, chronic	2/18 (11)	4/10 (40)	NS
Bacteremia	5/18 (28)	2/10 (20)	NS
Endocarditis	0/18 (0)	2/10 (20)	NS
Pyomyositis	2/18 (11)	0/10 (0)	NS
Liver abscess	1/18 (6)	0/10 (0)	NS
Pneumonia + empyema	6/18 (33)	1/10 (10)	NS
Septic joint	1/18 (6)	2/10 (20)	NS
Fasciitis	1/18 (6)	0/10 (0)	NS
Toxic-shock syndrome	1/18 (6)	0/10 (0)	NS
Other‡	3/18 (38)	0/10 (0)	NS
Patients with >1 disease manifestation (%)	7/18 (39)	1/10 (10)	NS
Days of illness before hospitalization, mean ± SD (range)	6.9 ± 11.8 (1–45)	10.9 d ± 15 (2–45)	NS
Days of positive cultures, mean ± SD (range)	6.94 ± 9.9 (1–45)	4.4 ± 2.98 (1–11)	NS
Initial leukocyte count§ (thousand/μL), mean ± SD (range)	14.8 ± 13.07 (2.4–57.0)	9.95 ± 4.45 (3.3–17.0)	NS

*CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus;* NS, not significant; SD, standard deviation. Among patients with invasive CA-MRSA infection, 1 died.
†Bonferroni correction for multiple comparisons was applied for comparisons of diagnosis.
‡Other diseases included extensive perineal abscess with fistula, subgaleal hematoma, and a subscapular abscess and prostatitis in the same patient.
§One initial leukocyte count was unavailable in each patient group.

patients with SSTI CA-MRSA disease, 12 were African American, and 2 were Hispanic. The average hospital stay for this group was 2.3 days. Seven children required no hospitalization or hospitalization <24 hours.

A representative PFGE result is depicted in the Figure. Two predominant clones emerged among the local and invasive CA-MRSA isolates. The clone A (n = 6) was identified to be of the USA400 lineage, and the B clone (n = 30) was identified to be of the USA300 lineage. No predominant clones emerged from the invasive MSSA isolates; 6 unique clones were identified from 10 isolates (data not shown). No isolates were of the USA300 or 400 lineage. The clinical manifestations of invasive disease in patients with CA-MRSA disease from clone A were pneumonia with empyema, osteomyelitis, bacteremia, and septic arthritis. The clinical manifestations of invasive disease in patients with clone B infection included sepsis, toxic-shock syndrome, osteomyelitis, fasciitis, bacteremia, pyomyositis, pneumonia with empyema, deep visceral abscesses, and perirectal abscess with prostatitis; 1 patient in this group died. Clone A was found in a wide geographic distribution around metropolitan Chicago. In contrast, clone B was located with more frequency within the city limits of Chicago.

Antimicrobial susceptibility patterns among CA-MRSA strains are detailed in Table 2. Two CA-MRSA isolates that were positive by 3-D test belonged to the A0 clone. None of the MRSA isolates that caused SSTI was D-test positive. The PVL gene was found in all of the CA-MRSA isolates that caused invasive and SSTI disease but in only 1 of 10 of the invasive CA-MSSA isolates ($p<0.001$).

Conclusions

PVL genes can be transmitted by means of bacteriophages, which allows them to be transmitted from 1 organism to another (*9*). When injected intradermally in rabbits, PVL induces necrotic skin lesions (*10*), and PVL has been described in *S. aureus* isolates from patients with necrotizing pneumonia, skin infections, and musculoskeletal infections. These outbreaks have been widespread (*1,2,8,11–13*). We found a wide range of severity of infection caused by clonally related CA-MRSA, PVL-positive isolates within our community, from superficial skin abscesses to fatal disease.

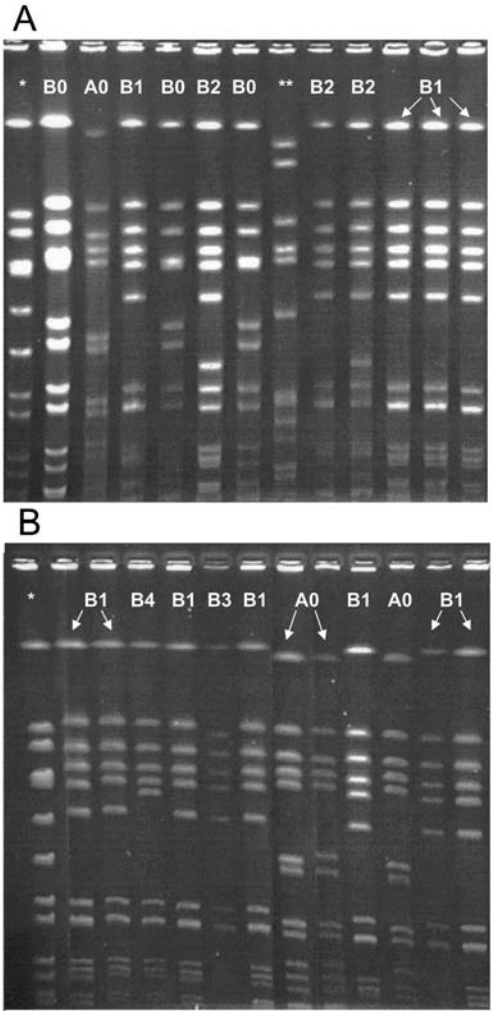

Figure. Pulsed-field gel electrophoresis (PFGE) results for community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) isolates causing disease. A) Local skin and soft tissue isolates. B) Invasive isolates. *, PFGE control; **, clinical isolate, hospital-associated MRSA.

Results of PFGE correlate well with results of other molecular typing methods, such as multilocus sequence typing (MLST), which characterizes *S. aureus* species by using sequences of 7 conserved housekeeping genes (*7*). In accordance with other published studies in the United States, the USA300 strain was the most frequently isolated among CA-MRSA disease (*12*) in contrast to the invasive CA-MSSA disease, which had no predominant clonality. We also detected isolates of the USA400 lineage that harbored PVL genes. This lineage was previously described as a cause of severe and fatal CA-MRSA disease in children in the Midwest (*14*). Previous reports from University of Chicago have described a cluster of 4 cases in which USA400 isolates caused empyema and sepsis syndrome with some features of toxic-shock syndrome (*4*); 3 children died with necrotizing pneumonia and Waterhouse-Friderichsen syndrome due to a PVL-positive, MLST-identified type 1 strain (*15*). In contrast, our patient with fatal toxic-shock syndrome did not have any primary pulmonary pathology and had disease caused by a USA300 lineage strain.

Limitations to our study include its retrospective nature and the limited numbers of patients. The geographic distribution of CA-MRSA isolates within the city likely reflects the geographic distribution of our patient population. Future prospective studies may further elucidate possible epidemiologic risk factors associated with acquiring CA-MRSA invasive infection.

Dr Jaggi is a pediatric infectious disease fellow at Children's Memorial Hospital (Northwestern). Her major research interests have included group A streptococcal infections and pediatric community-associated MRSA infections.

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Table 2. Susceptibility data for CA-MRSA isolates from children*

Susceptibility to antimicrobial agent†	CA-MRSA isolates causing invasive disease, n (%)	CA-MRSA isolates causing local skin/ soft tissue infections, n (%)	p value
Resistant to erythromycin	16/18 (88.9)	11/18 (61)	NS
Apparently susceptible to clindamycin	18/18 (100)	18/18 (100)	NS
Inducible clindamycin resistance	3/16 (19)	0/10 (0)	NS
Resistant to ciprofloxacin	2/18 (11)	1/18 (6)	NS
Resistant to levofloxacin	1/18 (6)	1/18 (6)	NS
Resistant to tetracycline	1/18 (5)	2/18 (11)	NS

*CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*; NS, not significant.
†In addition to the antimicrobial agents listed, all isolates were susceptible to vancomycin, linezolid, and rifampin.

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Address for correspondence: Tina Q. Tan, Children's Memorial Hospital, 2300 Children's Plaza, Box 20, Chicago, IL, USA 60614; email: titan@childrensmemorial.org

Ertapenem Resistance of *Escherichia coli*

Marie-Frédérique Lartigue,* Laurent Poirel,* Claire Poyart,† Hélène Réglier-Poupet,† and Patrice Nordmann*

An ertapenem-resistant *Escherichia coli* isolate was recovered from peritoneal fluid in a patient who had been treated with imipenem/cilastatin for 10 days. Ertapenem resistance may be explained by a defect in the outer membrane protein and production of extended-spectrum β-lactamase CTX-M-2.

Of all β-lactam antimicrobial drugs, carbapenems (imipenem, meropenem, and ertapenem) have the most consistent activity against *Enterobacteriaceae*. Activity is retained against most isolates that produce high-level AmpC β-lactamases (cephalosporinases) and clavulanic-acid–inhibited extended-spectrum β-lactamases (ESBL) (*1*). However, a few carbapenem-resistant enterobacterial isolates have been reported; resistance may be caused by production of carbapenemases (*2*) or by combined mechanisms of an outer membrane permeability defect and extended-spectrum β-lactamases or cephalosporinase (*3–6*). Spread of CTX-M type ESBLs, especially in *Escherichia coli*, may provide a favorable background for selection of carbapenem resistance. Resistance to the recently introduced ertapenem has not been reported in *E. coli* associated with a CTX-M-type enzyme. We describe the clinical and microbiologic features associated with an ertapenem-resistant *E. coli* isolate that had reduced susceptibility to imipenem after in vivo treatment with imipenem/cilastatin and provide a detailed molecular analysis of the antimicrobial drug resistance mechanisms.

The Study

E. coli CO strain was recovered from a 50-year-old immunocompromised woman who was hospitalized for a combined liver and heart transplant. She had a history of cardiac failure, hepatitis C virus–related liver cirrhosis, and chronic renal insufficiency. After surgery, septic shock developed related to perforation of the colon. The patient received a full dose of imipenem/cilastatin (2 g/day), a reduced dose of vancomycin (400 mg/day), gentamicin (100 mg/day for 2 days), and fluconazole (100 mg/day). Ten days later, peritoneal lavage and surgery to remove diseased colonic tissue were performed, but the patient died 2 days after surgery. Culture of the peritoneal fluid yielded an ertapenem-resistant *E. coli* CO strain.

Disk diffusion susceptibility testing with antimicrobial drug–containing disks (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) (*7*) was performed with and without cloxacillin (250 mg/L), which is a β-lactam molecule that inhibits in vitro cephalosporinase activity (*5*). MICs were determined by an agar dilution technique and interpreted according to Clinical and Laboratory Standards Institute guidelines (*7*). The *E. coli* CO strain was resistant to extended-spectrum cephalosporins, cefoxitin, and moxalactam. In addition, it was intermediately susceptible to imipenem and meropenem (MIC 8 mg/L each) and was resistant to ertapenem (MIC >256 mg/L) (Table). The *E. coli* CO strain was also resistant to gentamicin, kanamycin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole; intermediately susceptible to nalidixic acid and tobramycin; and remained susceptible to amikacin, netilmicin, ofloxacin, and ciprofloxacin. Antimicrobial drug susceptibility testing on cloxacillin-containing plates indicated absence of consequential cephalosporinase activity. However, the ceftazidime/clavulanic acid synergy test result was slightly positive. A β-lactamase extract from a culture of *E. coli* CO subjected to isoelectric focusing analysis showed 3 β-lactamase activities with pI values of 5.4, 6.1, and 7.9 (*8*). This extract did not hydrolyze carbapenems according to spectrophotometer measurements (*8*). Conjugation experiments that used an azide-resistant *E. coli* J53 strain as recipient strain (*5*), followed by selection on Mueller-Hinton agar plates containing 100 mg/L sodium azide and 100 mg/L amoxicillin or 2 mg/L of cefotaxime, yielded transconjugants. Two conjugative plasmids (pCO-1, 160 kb; pCO-2, 150 kb) were extracted from those transconjugants by the Kieser technique (*5*). They conferred resistance to amoxicillin and ticarcillin, whereas pCO-1 conferred additional resistance to extended-spectrum cephalosporins (Table). These transconjugants were fully susceptible to carbapenems. Standard PCR conditions were used to amplify several β-lactamase genes coding for carbapenemases (bla_{KPC}, bla_{NMC-A}); extended-spectrum β-lactamases including bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, bla_{PER}; and oxacillinases (OXA-1, OXA-2, OXA-10, OXA-21, and OXA-48) (*2*,*9*). PCR amplification and sequencing identified an extended-spectrum β-lactamase $bla_{CTX-M-2}$ gene located on plasmid pCO-1, whereas a bla_{TEM-1} gene that coded for narrow-spectrum penicillinase and a bla_{OXA-10} gene that coded for oxacillinase were both located on a 150-kb plasmid pCO-2. The surrounding regions of the $bla_{CTX-M-2}$ gene corresponded to those of a *sul1*-type class 1 integron. This gene was bracketed by a

*Université Paris XI, Kremlin-Bicêtre, France; and †Université Paris V, Paris, France

Table. MICs of β-lactam antimicrobial drugs for *Escherichia coli* CO, transconjugants pCO-1 and pCO-2, and reference strain *E. coli* J53*

β-lactam	MIC (mg/L)			
	E. coli CO	Transconjugant pCO-1 (CTX-M-2)	Transconjugant pCO-2 (OXA-10, TEM-1)	*E. coli* J53
Amoxicillin	>256	>256	>256	2
Amoxicillin + CLA	>256	8	64	2
Ticarcillin	>256	>256	>256	2
Ticarcillin + CLA	>256	32	128	2
Piperacillin	>256	>256	64	1
Piperacillin + TZB	256	2	32	1
Cephalotin	>256	>256	4	4
Cefoxitin	256	4	4	4
Ceftazidime	64	4	0.06	0.06
Ceftazidime + CLA	1	0.125	0.06	0.06
Cefotaxime	>256	64	0.06	0.06
Cefotaxime + CLA	128	1	0.06	0.06
Cefepime	>256	16	0.06	0.06
Moxalactam	128	0.06	0.06	0.06
Aztreonam	>256	32	0.06	0.06
Imipenem	8	0.25	0.25	0.25
Meropenem	8	0.06	0.06	0.06
Ertapenem	>256	0.03	0.03	0.03

*CLA, clavulanic acid at a fixed concentration of 2 mg/L; TZB, tazobactam at a fixed concentration of 4 mg/L..

duplication of the 3′-conserved sequence region of the class 1 integron and was not associated with a 59-bp element. The common region open reading frame (ORF) 513 was found upstream of the $bla_{\text{CTX-M-2}}$ gene (data not shown) (*10*).

The outer membrane protein (OMP) profiles of *E. coli* isolates were extracted and analyzed by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as described (*4*,*5*,*11*,*12*) and compared with profiles of *E. coli* control strains expressing porins OmpC or OmpF (*13*). The OMP profiles of *E. coli* CO showed expression of OmpA and OmpF and no expression of OmpC (Figure). Using whole-cell DNA of *E. coli* CO as a template and primers EcOmpFA (5′-CAGGTACTGCAAACGCTGC-3′) and EcOmpFB (5′-GTCAACATAGGTGGAC ATG-3′) that anneal at the ends of the *ompF* gene of *E. coli* (*5*), we obtained a 953-bp internal fragment of the *ompF* gene (data not shown). Sequencing identified a wild-type *ompF* gene. When primers EcOmpCA (5′-GTTAAAGTACT GTCCCTCCTG-3′) and EcOmpCB (5′-GAACTG GTAAACCAGACCCAG-3′) were used, no amplification was obtained for *E. coli* CO, whereas a 1,086-bp internal fragment of the *ompC* gene of the *E. coli* control strain expressing OmpC (*10*) and of 3 wild-type *E. coli* strains was amplified (data not shown). Thus, the *ompC* gene was either disrupted or not present, which explains lack of expression of this protein and might contribute substantially to ertapenem resistance of *E. coli* CO.

Conclusions

Ertapenem resistance has been reported in *Klebsiella pneumoniae*–producing CTX-M–type ESBLs that have a permeability defect (*3*,*4*,*14*). We report here the first ertapenem-resistant *E. coli* clinical isolate that produced a CTX-M-type ESBL and that was deficient in porin OmpC. This finding may be clinically relevant because ertapenem is approved for treatment of peritonitis, abdominal infection, and complicated skin and soft tissue infections in

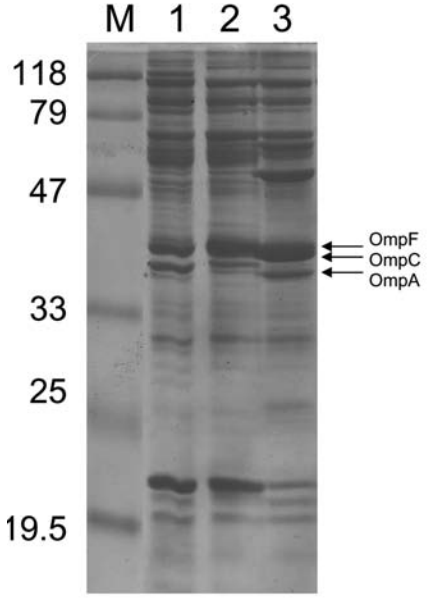

Figure. Outer membrane protein (OMP) profiles of *Escherichia coli* strains. OMP content was determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1 corresponds to *E. coli* CO clinical isolate; lane 2, *E. coli* JF 568 strain expressing OmpC; lane 3, *E. coli* JF 701 strain lacking OmpC (*9*). The molecular mass marker (M) and corresponding sizes (in kilodaltons) are indicated on the left. Horizontal arrows on the right indicate positions of the OMPs OmpF, OmpC, and OmpA.

patients with diabetes and because *E. coli* is the main species isolated in human infections and the main enterobacterial species that expresses these emerging extended-spectrum β-lactamases CTX-M (*15*). That an imipenem/cilastatin–containing regimen was likely able to select for ertapenem resistance is cause for concern. Moreover, even if the strain is not resistant to imipenem and meropenem, it is no longer totally susceptible. Susceptibility patterns of this *E. coli* CO strain, although resistant to ertapenem, are also highly resistant to extended-spectrum cephalosporins, thereby demonstrating an ESBL phenotype. This information may help with future identification of those multidrug CTX-M (+) resistance isolates for which the best treatment remains carbapenems.

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Dr Lartigue is a microbiologist at the Université Paris XI. She recently completed her PhD thesis on emerging and genetic trends of β-lactam resistance genes.

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Address for correspondence: Patrice Nordmann, Service de Bactériologie-Virologie, Hôpital de Bicêtre, 78 Rue du Général Leclerc, 94275 Kremlin-Bicêtre, France; email: nordmann.patrice@bct.aphp.fr

Surveillance for Shiga Toxin–producing *Escherichia coli*, Michigan, 2001–2005

Shannon D. Manning,* Robbie T. Madera,† William Schneider,† Stephen E. Dietrich,† Walid Khalife,‡ William Brown,§ Thomas S. Whittam,* Patricia Somsel,† and James T. Rudrik†

A surveillance system used different detection methods to estimate prevalence of Shiga toxin–producing *Escherichia coli* during 2003–2005 and 2001–2002. More non-O157 serotypes were detected by enzyme immunoassay than by evaluation of non–sorbitol-fermenting *E. coli* isolates. We therefore recommend use of enzyme immunoassay and culture-based methods.

Infection with Shiga toxin–producing *Escherichia coli* (STEC) is a frequent cause of gastrointestinal disease, particularly among children and elderly persons (*1*). Detection of O157 STEC by culture relies primarily on sorbitol MacConkey agar (SMAC) (*2*) because O157:H7 strains cannot rapidly ferment sorbitol (*3*). In contrast, using culture to detect sorbitol-fermenting O157 (*4*) and non-O157 serotypes is problematic because on SMAC these strains are indistinguishable from other *E. coli*. Consequently, whether the predominance of STEC O157 in disease reflects actual differences in pathogen prevalence or a bias associated with detection is unclear. We therefore sought to determine whether STEC prevalence, particularly of non-O157 serotypes, increased when enhanced detection methods were used.

The Study

The Michigan Department of Community Health implemented a sentinel surveillance system to evaluate blood-containing stool samples from 20 laboratories during April 2003–October 2005 and all stool samples from 2 hospitals during July 2004–October 2005. All suspect non–sorbitol-fermenting *E. coli* from the remaining laboratories were also examined.

*Michigan State University, East Lansing, Michigan, USA; †Michigan Department of Community Health, Lansing, Michigan, USA; ‡Sparrow Health System, Lansing, Michigan, USA; and §Detroit Medical Center, Detroit, Michigan, USA

The samples, transported in C&S transport medium (Medical Chemical Corporation, Torrance, CA, USA), were screened for Shiga toxin (Stx) by enzyme immunoassay (EIA) (Meridian BioScience, Cincinnati, OH, USA) after enrichment with gram-negative broth (Remel, Lenexa, KS, USA). EIA is sensitive and specific but cannot detect the Stx2e variant (*5*), and *Pseudomonas aeruginosa* can produce false-positive results (*6*). Samples were cultured on SMAC (Remel) and cefixime-tellurite SMAC (*7*), and samples from the 2 hospitals were tested for occult blood (Beckman Coulter, Fullerton, CA, USA) before EIA testing. Serotyping (Statens Serum Institute, Copenhagen, Denmark; BD Difco, Franklin Lakes, NJ, USA) and real-time PCR for *stx1,2* genes (*8*) were performed on strains that had positive EIA results, suspect non–sorbitol-fermenting *E. coli*, and multiple colonies of sorbitol-fermenting (SF) strains that had positive EIA results. For some samples, the EIA result was negative but NSF *stx*-positive colonies were detected on SMAC, which indicated a false-negative EIA result. Epidemiologic data were obtained for STEC-positive patients.

During the 5 years studied, 438 STEC were isolated; 401 (92%) were O157. Prevalence over time did not differ ($\chi^2 = 4.14$, degrees of freedom [df] = 4, p = 0.39). Similarly, overall prevalence of non-O157 serotypes during 2001–2002 and 2003–2005 did not differ ($\chi^2 = 0.83$, df = 1, p = 0.36). Most (70%) STEC isolates were recovered between June and October from heavily populated areas (Figure). No SF O157 were recovered.

In 2001–2002, a total of 664 suspect NSF *E. coli* isolates were evaluated; 179 (27%) were O157 and 2 (0.3%) were non-O157 serotypes (Table 1). After enhanced surveillance began in 2003, a total of 852 suspect isolates were tested; 177 (21%) were O157 and 3 (0.4%) were non-O157 serotypes (Table 1). The remaining STEC (n = 28, 2001–2002; n = 49, 2003–2005) were detected by EIA. For 5 samples, EIA yielded a false-negative result but STEC

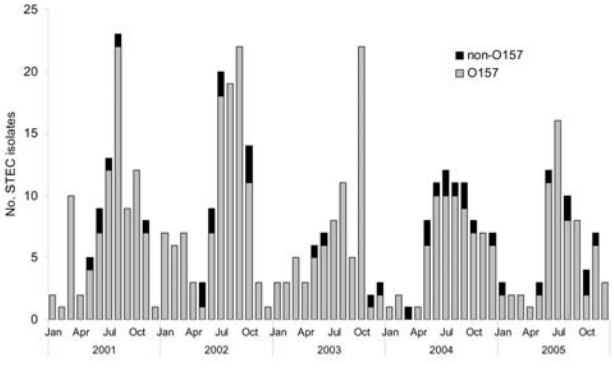

Figure. Frequency of isolation of Shiga toxin–producing *Escherichia coli* (STEC), Michigan, 2001–2005. Enhanced surveillance for STEC began in April 2003.

Table 1. Evaluation of shiga toxin–producing *Escherichia coli* detection methods, Michigan, 2001–2005*

Detection method	Stool samples	Routine STEC surveillance†, no. (%)		Enhanced STEC surveillance, no. (%)		
		2001	2002	2003	2004	2005
Culture and PCR‡ for *stx* genes of suspect NSF *E. coli*	Total	327	337	249	317	286
	O157	85 (26)	94 (28)	63 (25)	58 (18)	56 (20)
	Non-O157	2 (0.6)	0 (0)	0 (0)	2 (0.6)	1 (0.3)
EIA of bloody or suspect samples	Total	N/A	N/A	209	141	73
	O157	4	11	11 (5)	7 (5)	6 (8)
	Non-O157	4	9	4 (2)	7 (5)	7 (10)
EIA of all samples	Total	N/A	N/A	N/A	1,405	1,021
	O157	N/A	N/A	N/A	5 (0.4)	1 (0.1)
	Non-O157	N/A	N/A	N/A	1 (0.07)	0 (0)

*STEC, Shiga toxin–producing *Escherichia coli*; NSF, non–sorbitol-fermenting; EIA, enzyme immunoassay.
†In 2001 and 2002, EIA of bloody stools was performed by 2 large clinical laboratories in Michigan and was not part of the enhanced STEC surveillance system at the Michigan Department of Community Health. Therefore, the total number of EIAs performed is not known; the total number of EIA s submitted as positive for Stx is provided by serotype.
‡PCR for *stx1,2* genes was not initiated at the Michigan Department of Community Health until 2002. Before 2002, a DNA probe (Digene Signal Kit, Digene Diagnostic, Inc., Silver Spring, MD, USA) was used to detect STEC.

were recovered from SMAC. During 2003–2005, 7 of the 49 STEC came from screening all 2,426 samples from the 2 hospitals; 359 (15%) of these samples contained occult blood. Among the 7 detected, 5 contained occult blood and 6 were O157. The remaining 42 (10%) STEC were found by screening 423 suspect samples from other laboratories; 18 (4%) were non-O157 serotypes. More non-O157 serotypes were detected (χ^2 = 61.1, df = 1, p<0.00001) from 2003–2005 when EIA was used instead of the NSF *E. coli* isolate submission process. Among all 37 non-O157 serotypes isolated, O45:H2 (n = 10) and O26:H11 (n = 5) predominated.

Epidemiologic data were available for up to 389 (89%) STEC patients, depending on the variable assessed. Most patients (62%) were Caucasian; ≈50% were female. Disease occurred mostly in persons ≤10 (27%), 11–18 (19%), and 19–30 (17%) years of age. Although disease frequency was lower (9%) in persons >65 years of age, these persons were more likely to be hospitalized than were persons ≤18 years of age, as were persons with bloody diarrhea or hemolytic-uremic syndrome (HUS) (Table 2). Among the 12 patients with HUS, 2 were infected with non-O157 serotypes O103:H2 and O76:H7, and 7 of the 12 HUS-associated strains were *stx2* only.

To adjust for factors associated with hospitalization, we fit a logistic regression model that included age and symptom variables in the model. The adjusted associations were similar to the crude associations. Hospitalization was more frequent for persons with bloody diarrhea (adjusted odds ratio [OR] 1.8, 95% confidence interval [CI] 1.04–3.08) and HUS (adjusted OR 16.0, 95% CI 2.00–127.47). Also, persons 19–64 (adjusted OR 1.6, 95% CI 1.05–2.59) and >65 (adjusted OR 6.6, 95% CI 2.57–17.15) years of age were hospitalized more frequently than persons ≤18 years of age.

Conclusions

Enhanced detection methods did not significantly increase the year-to-year recovery of STEC. Overall, the observed STEC prevalence decreased slightly over time, similar to the national trend of an overall 42% decrease in STEC O157 incidence during 1996–2004 (*9*). This reduction is likely attributable to numerous factors, including heightened consumer awareness (*9*) and improved screening protocols during food production (*10*).

Enhanced surveillance did, however, enhance detection of non-O157 serotypes; 4.3% of EIA-positive stools were non-O157 compared with 0.5% of suspect NSF *E. coli*. Additionally, among the STEC found, 34 (48%) were non-O157 and 37 (52%) were O157 when EIA was used on suspect stools, compared with only 3 (1.6%) non-O157 and 177 (98.3%) O157 among NSF *E. coli*. Despite enhanced surveillance, STEC prevalence is probably still underestimated, particularly for non-O157 serotypes, because not all ill persons seek medical care and not all laboratories submit suspect stools for evaluation. Nevertheless, in 5 years, our surveillance identified 66 (15%) cases that would have been undetected by conventional methods; 31 (47%) were non-O157. Among those patients for whom data were available, 27 (42%) of 64 were <18 years of age, 22 (43%) of 51 were hospitalized, and 39 (76%) of 51 had bloody diarrhea. Although bloody stool and patient age are poor predictors of STEC infection (*11*), our analysis demonstrates that screening bloody stool samples improves detection of non-O157, and blood and older age are important predictors of more severe disease, which may be more costly if undetected.

Hospitalization of STEC patients with and without HUS costs an estimated US $30,307 and $4,061 per patient, respectively (*12*). Therefore, Michigan hospital costs associated with STEC infection likely exceeded

Table 2. Association of characteristics with infection by Shiga toxin–producing *Escherichia coli* among 389 of 438 patients for whom data were available, Michigan, 2001–2005*†

Characteristic	No. with characteristic	No. (%) hospitalized	OR (95% CI)
Demographics			
Age, y			
≤18	178	77 (43)	1.0
19–64	178	94 (53)	1.5 (0.95–2.28)
≥65	32	26 (81)	5.7 (2.09–16.27)
Sex			
Female	204	105 (51)	1.0
Male	185	92 (50)	0.9 (0.61–1.42)
Clinical symptoms			
Abdominal pain			
No	76	40 (53)	1.0
Yes	304	151 (50)	0.9 (0.54–1.47)
Body aches			
No	323	161 (50)	1.0
Yes	57	30 (53)	1.1 (0.64–1.96)
Chills			
No	313	155 (50)	1.0
Yes	66	36 (55)	1.2 (0.72–2.08)
Diarrhea			
No	154	82 (53)	1.0
Yes	226	109 (48)	0.8 (0.54–1.23)
Bloody diarrhea			
No	77	30 (39)	1.0
Yes	303	161 (53)	1.8 (1.10–2.96)
HUS			
No	368	180 (49)	1.0
Yes	12	11 (92)	11.5 (1.47–89.90)
Bacterial serotype and genes			
O157	360	181 (50)	1.0
Non-O157	29	16 (55)	1.2 (0.57–2.60)
stx1	28	14 (50)	1.0
stx2	121	54 (45)	0.8 (0.33–1.98)
stx1,2	239	128 (54)	1.2 (0.49–2.70)

*OR, odds ratio; CI, confidence interval; HUS, hemolytic-uremic syndrome.
†Some numbers do not add up to the total (n = 389) because of missing values (never >2.6%).

$1,119,050 during 2001–2005, as 198 patients were hospitalized and 12 had HUS. Identification of each additional STEC case could have a substantial public health effect in that 1 case may lead to the recognition of an outbreak, which if detected early, could contribute to a cost savings as well as reduced STEC-associated illness. We estimated that the cost to detect each of the 66 additional cases using the EIA ($7 per test including labor) differed considerably when we evaluated screening of all stool samples ($2,426/per positive) versus suspect stool samples ($10/per positive).

No widely available test detects all STEC, and use of multiple methods is not cost-effective. Consequently, we recommend using EIA in conjunction with SMAC culture to recover isolates for molecular characterization and subsequent outbreak investigations. Although occult blood tests did not enhance the sensitivity of STEC recovery, patient data and accompanying epidemiologic information may help identify which samples to test, thereby preventing future outbreaks. Because such epidemiologic information is often not available to laboratory personnel, we suggest that clinical laboratories work with medical administrations to use EIAs as their standard of practice and to facilitate routine availability of such information. Until more sensitive and cost-effective STEC screening methods are available, facilities that cannot implement EIAs should forward stool samples that are suspect, as well as those with positive screening results, to public health laboratories. These laboratories can easily evaluate suspect stools for STEC by EIA or PCR followed by culture of all positive samples to recover the isolate for further characterization.

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Dr Manning is a molecular and infectious disease epidemiologist. She studies the molecular epidemiology of pathogenic *E. coli* and group B *Streptococcus* at Michigan State University.

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Address for correspondence: James T. Rudrik, Michigan Department of Community Health, Bureau of Laboratories, 3350 North Martin Luther King Jr Blvd, Bldg 44, Rm 129, Lansing, MI 48909, USA; email: RudrikJ@michigan.gov

Rapid Genome Sequencing of RNA Viruses

Tetsuya Mizutani,* Daiji Endoh,† Michiko Okamoto,‡ Kazuya Shirato,* Hiroyuki Shimizu,* Minetaro Arita,* Shuetsu Fukushi,* Masayuki Saijo,* Kouji, Sakai,* Chang Kweng Lim,* Mikako Ito,* Reiko Nerome,* Tomohiko Takasaki,* Koji Ishii,* Tetsuro Suzuki,* Ichiro Kurane,* Shigeru Morikawa,* and Hidekazu Nishimura‡

We developed a system for rapid determination of viral RNA sequences whereby genomic sequence is obtained from cultured virus isolates without subcloning into plasmid vectors. This method affords new opportunities to address the challenges of unknown or untypeable emerging viruses.

Over the past few years, global migration has led to emerging infectious diseases that pose substantial risks to public health. To prevent potential outbreaks, early detection of infectious pathogens is necessary. In particular, the recent outbreak of severe acute respiratory syndrome (SARS) provided important lessons on how unknown viruses should be detected rapidly. Thus, a standardized and qualified system is required for rapid nucleic acid sequence determination for newly emerging viruses.

Recently, we developed a new method for detecting RNA viruses. This method, based on cDNA representational difference analysis (cDNA RDA), uses 96 hexanucleotides that are not suitable for priming ribosomal RNAs but that normally prime most of the genome of an RNA virus as primers for reverse transcription in cDNA RDA (*1*). However, the RDA method with a cloning step requires at least 1 week for the determination of the nucleic acid sequence.

The Method

Our new system for rapid determination of viral RNA sequence (RDV) uses whole-genome amplification and direct sequencing techniques (Figure 1). The RDV method comprises 6 procedures: 1) effective destruction of cellular RNA and DNA for semipurification of viral particles, 2) effective elimination of DNA fragments by using a prefiltration column system and elution of small amounts of RNA, 3) effective synthesis of first- and second-strand cDNAs, 4) construction and amplification of a cDNA library, 5) construction of a second cDNA library, and 6) direct sequencing using optimized primers. The RDV method enables a broad range of partial nucleotide sequences within the entire viral RNA genome to be obtained within 2 days without cloning into plasmids.

To eliminate contaminating cellular RNA and DNA from the samples, 0.001 μg of RNase A (Qiagen, Hilden, Germany) and 1 μL (2 U) of Turbo DNA-free DNase I (Ambion, Austin, TX, USA) with 1× Turbo DNA-free buffer were incubated at 37°C for 30 min under conditions that prevented destruction of viral RNA in the viral particles. The RNA in the viral particles was then extracted within 30 min by using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). We confirmed that DNA was effectively eliminated by this RNA extraction kit.

In accordance with the Invitrogen manual, cDNA was synthesized, by using random hexamers (Takara Bio Inc., Kyoto, Japan) and Superscript III (Invitrogen, Carlsbad, CA, USA) lacking RNase H activity, at 50°C for 1 h. Then 60 U of RNase H (Takara Bio Inc.) added before synthesis of second-strand cDNA at 50°C for 1 h. In accordance with the manual, a whole genome amplification system (WGA; Sigma-Aldrich, Saint Louis, MO, USA), which was developed for amplification of genomic DNA, was used to amplify viral double-stranded cDNA. This process was

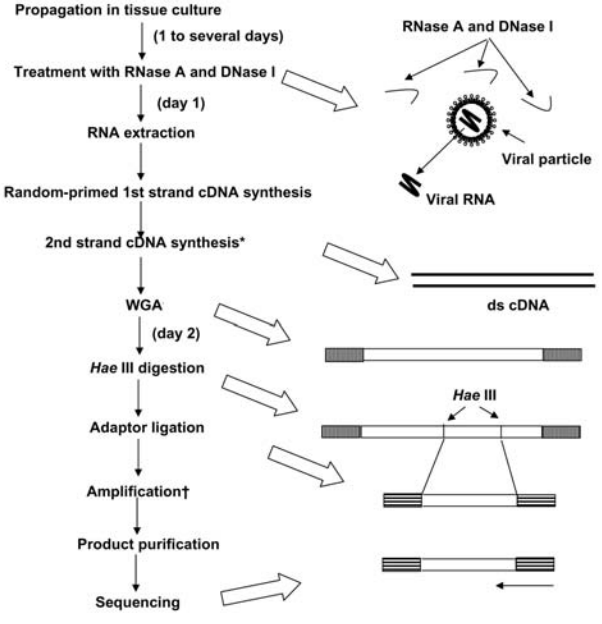

Figure 1. Overall scheme of the rapid determination of viral RNA sequence method. *By adding RNase H; WGA, whole genome amplification; †With specially designed primer sets as shown in Figure 2.

*National Institute of Infectious Diseases, Tokyo, Japan; †Rakuno Gakuen University, Ebetsu, Japan; and ‡Sendai Medical Center, Sendai, Japan

performed within 90 min. Instead of the Taq polymerase recommended in the kit, we used 1.25 U of AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the PCR products. Primers were provided in the WGA kit, but no information regarding their sequences was obtained. The reaction mixture was heated at 95°C for 9 min (for activation of AmpliTaq Gold), followed by 70 cycles of amplification using Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 68°C for 1 min, primer extension at 72°C for 5 min, and denaturation at 94°C for 1 min.

The 1st cDNA library was digested with 40 U of *Hae*III (Takara Bio Inc.) at 37°C for 30 min. DNA was purified by using the MonoFas DNA isolation system (GL Science, Tokyo, Japan), and a blunt *Eco*RI-*Not*I-*Bam*HI adaptor (10 pmol; Takara Bio Inc.) was ligated at 16°C for 30 min by using DNA Ligation Kit, Mighty Mix (Takara Bio Inc.). The second cDNA library was amplified by PCR with specially designed primer sets in which 6 nucleotides composed of CC (*Hae*III-digested sequence) and 4 variable nucleotides were added to the 3′ end of the adaptor sequence (Figure 2). For example, 1 primer set was as follows: forward primer, H1–1: 5′-AATTCGGCGGCCGCGGATCC*CC*GGGG-3′; reverse primer H9–3: 5′-AATTCGGCGGCCGCGGATCC*CC*AGGA-3′ (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics) (Figure 2).

We always used >12 primer sets and 0.83 μmol of each primer per cDNA library. PCR was performed with AmpliTaq Gold Master Mix (Applied Biosystems). The reaction mixture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing and primer extension at 72°C for 30 s and denaturation at 94°C for 30 s. A single band was consistently obtained in ≈50% of the reactions. DNA was purified from the PCR by using MonoFas. Occasionally, we purified DNA fragments from the gels when >2 bands were detected. Direct sequencing was performed with the forward primer, reverse primer, or both.

When the number of viral particles in the sample was high, we omitted the RNase A and DNase I treatments and used the RNeasy Mini Kit (Qiagen) for RNA extraction. We occasionally used a whole transcriptome amplification kit (Rubicon Genomics Inc, Ann Arbor, MI, USA) instead of the WGA kit because both kits yielded similar amplification results.

In preliminary studies that used referential RNA viruses, we attempted to determine the nucleic acid sequences of SARS coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants (10–100 μL) by using the RDV method. The percentages of positive fragments (number of fragments containing viral nucleic acid/total number of sequenced fragments) in the reactions for detection of these 5 viruses were 60% (3/5), 45% (5/11), 100% (12/12), 50% (5/10), and 40% (4/10), respectively. As a clinical application, a throat swab specimen from a patient with fever and upper respiratory infection was characterized. Although the specimen exhibited enterovirus-like cytopathic effect by inoculation into HEF and GMK cells when cell culture system for virus isolation was used (*2*), extracted RNA from the supernatant of the cells showed no amplification by reverse transcription–PCR (RT-PCR) when 1 of the conventional primer sets for human enteroviruses was used (*3*,*4*). In the cell culture supernatant analysis by the RDV method, the specimen exhibited amplification of the partial nucleotide sequences of coxsackie A14 virus (nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB275848–AB275853). Thus, the RDV method could detect unidentified cytopathic-effect agents such as enterovirus that could not be detected by RT-PCR when the conventional primer set for enteroviruses was used.

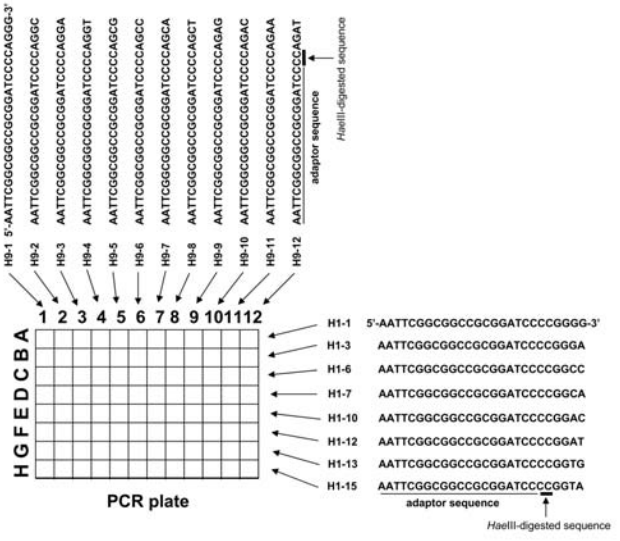

Figure 2. Primers used in rapid determination of viral RNA sequence method.

Conclusions

The RDV method is a rapid method for the direct determination of viral RNA sequences without using the cDNA cloning procedure. The limitations of the RDV method are the requirement for cell culture isolate and the large number of steps. However, RDV would be useful for species-independent detection of RNA viruses including unknown or untypeable emerging RNA viruses. Furthermore, with minor modifications, this method would also be applicable to the detection of DNA viruses and bacteria.

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Dr Mizutani is a senior researcher at the National Institute of Infectious Diseases, Tokyo, Japan. His current research focus is infectious disease surveillance by using new technologies.

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Address for correspondence: Tetsuya Mizutani, Department of Virology 1, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama City, Tokyo 208-0011, Japan; email: tmizutan@nih.go.jp

Pneumocystis Pneumonia in HIV-positive Adults, Malawi[1]

Joep J.G. van Oosterhout,* Miriam K. Laufer,† M. Arantza Perez,* Stephen M. Graham,* Nelson Chimbiya,* Phillip C. Thesing,* Miriam J. Álvarez-Martinez,‡ Paul E. Wilson,‡ Maganizo Chagomerana,* Eduard E. Zijlstra,* Terrie E. Taylor,*§ Christopher V. Plowe,† and Steven R. Meshnick‡

In a prospective study of 660 HIV-positive Malawian adults, we diagnosed *Pneumocystis jirovecii* pneumonia (PcP) using clinical features, induced sputum for immunofluorescent staining, real-time PCR, and posttreatment follow-up. PcP incidence was highest in patients with the lowest CD4 counts, but PcP is uncommon compared with incidences of pulmonary tuberculosis and bacterial pneumonia.

The incidence of *Pneumocystis jirovecii* pneumonia (PcP) in HIV-infected adults in the sub-Saharan African region remains uncertain. That PcP is common in African children <1 year of age is well documented (*1*), but reported prevalence and incidence rates in adult African populations vary widely (*2*). Many of these reports were cross-sectional studies in selected populations from tertiary hospitals (*3*–*5*), and therefore might contain selection bias that favors identifying higher rates of PcP.

To our knowledge, no large prospective studies have been done by using broncho-alveolar lavage (BAL) in combination with immunofluorescent (IF) staining for *P. jirovecii* cysts, the diagnostic procedures of choice. Real-time PCR performed on sputum samples has high sensitivity but low specificity for PcP (*6*,*7*). The few studies in African adults that used PCR assays for *Pneumocystis* did not distinguish subclinical colonization from infection, mainly because of limited follow-up after diagnosis (*3*,*4*). We describe here the incidence of PcP from a large cohort study of HIV-infected Malawian adults that used a comprehensive diagnostic approach that included induced sputum with IF staining, real-time PCR, and follow-up after diagnosis and treatment.

The Study

HIV-infected adults (>15 years of age), who sought treatment at a government health center in the township of Ndirande, Blantyre, Malawi, were enrolled in a prospective, community-based study to determine the incidence of infections that were preventable by trimethoprim-sulfamethoxazole prophylaxis (*8*). Clinical evaluations were performed monthly and at sick visits occurring between the scheduled monthly evaluations. CD4 counts were determined every 4 months. Standardized diagnostic and treatment guidelines and case definitions were used. At the time of the study, in Malawi, antiretroviral therapy (ART) was rarely used, and trimethoprim-sulfamethoxazole prophylaxis was not recommended.

Cases of suspected PcP were identified by patients' clinical signs and symptoms, chest x-ray results, oxygen desaturation exercise test results (*9*), CD4 count, and failure to improve with antimicrobial treatment without activity against *P. jirovecii*. Patients' sputum production was induced by an ultrasonic nebulizer with hypertonic saline, followed by IF staining for *P. jirovecii* cysts. A case was classified as clinical PcP when the IF staining for *P. jirovecii* cysts was positive or the participant had strong clinical evidence of PcP and negative IF. Clinical follow-up data were collected after the episode of suspected PcP.

After the study, real-time PCR for the *P. jirovecii* dihydropteroate synthase and human RNAase P (control DNA) was performed on DNA extracted from the stored induced sputum slides (*10*). Clinicians were not aware of the PCR results during the study, and laboratory staff performing the PCR was blinded to clinical information and IF results. A final diagnosis of confirmed PcP was made for any episode with a positive IF result, positive PCR result, or both, unless recovery (defined as resolution of respiratory symptoms present at the start of the episode) without PcP treatment was observed with a minimum of 4 weeks of follow-up. If the PCR results were positive but the patient recovered without active treatment against PcP, the result was interpreted as *Pneumocystis* colonization. A negative PCR result ruled out PcP diagnosis in patients who had received PcP treatment on the basis of clinical evidence alone.

Incidence rates of respiratory diagnoses per 100 person-years of follow-up were calculated with 95% confidence intervals (CIs) based on Poisson distribution. First and subsequent episodes in the same person were counted separately, except for PcP, because patients with PcP

*University of Malawi College of Medicine, Blantyre, Malawi; †University of Maryland School of Medicine, Baltimore, Maryland, USA; ‡University of North Carolina, Chapel Hill, North Carolina, USA; and §Michigan State University, East Lansing, Michigan, USA

[1]Data from this study were presented in part at the 9th College of Medicine Research Dissemination Conference, Blantyre, Malawi, 12 Nov 2005 (abstract DCON/05/44), and the XV International AIDS Conference, Bangkok, Thailand, 11–16 Jul 2004 (abstract MoPeB3198).

received secondary prophylaxis and exited the study. The CD4 count at the time of the episode or within the previous 6 months was used for analysis.

We used χ^2, Mann-Whitney, and Student *t* tests for analysis of age, sex, and CD4 counts among diagnoses, respectively, using SPSS version 12 software (SPSS Inc., Chicago, IL, USA). The study was approved by the Institutional Review Boards of the University of Malawi College of Medicine, the University of Maryland, and Michigan State University.

Beginning in September 2002, 660 adults were enrolled in the study and followed up through August 2004. Baseline CD4 and World Health Organization stage data are shown in Table 1. Mean age was 31.7 years (range 16–66); 437 (66%) were female. Mean duration of follow up was 10.7 months (95% CI 10.4–11.5) per person. Eighty-six (13%) participants died, and 37 (6%) were withdrawn from the study because they started lifelong trimethoprim-sulfamethoxazole prophylaxis. Sixty-three participants (9.5%) left the area, 20 (3%) withdrew consent, and 119 (17%) were lost to follow up. A smaller proportion of patients from the lower CD4 strata exited the study than from the higher CD4 content group.

Ninety-five episodes of suspected PcP occurred in 75 persons. Outcomes of these episodes are given in Table 2.

Table 1. Baseline characteristics of study participants at enrollment, Malawi, 2002–2004*

Characteristic	No. episodes	PYO
CD4 (cells/mm^3)		
0–99	125	83
100–199	159	145
200–499	271	268
≥500	77	75
Missing at enrollment	28	19
HIV clinical stage†		
I	267	255
II	191	190
III	160	120
IV	42	25
Total	660	591

*PYO, person-years of observation (based on enrollment characteristics).
†As defined by World Health Organization criteria.

A final diagnosis of confirmed PcP was made in 6 episodes, and 9 episodes of *Pneumocystis* colonization were recorded, with a mean follow up of 26 weeks (range 4–48 weeks). Table 3 shows the incidence rates of PcP and other respiratory conditions in the cohort.

With full diagnostic workup including posttreatment follow up as the gold standard for the diagnosis of PcP, the sensitivity of PCR alone was 100%, the specificity 88%, and the positive predictive value 31%. Among episodes in which PcP was suspected, the mean CD4 count in patients

Table 2. Outcomes in 95 episodes of suspected *Pneumocystis* pneumonia (PcP), Malawi, 2002–2004*

Final diagnosis†	Clinical diagnosis‡	No. episodes	IF	PCR	Follow-up data
Confirmed PcP	PcP	2	Pos	Pos	–
Confirmed PcP	PcP	1	Neg	Pos	–
Confirmed PcP	PcP	1	–	–	Death after 2 wk of PcP treatment (IS not done due to respiratory distress)
Confirmed PcP	Bronchiectasis	1	Neg	Pos	Death 1 wk after IS
Confirmed PcP	Tuberculosis	1	Neg	Pos	Improvement but short follow-up (2 wk)
Pneumocystis colonization/ pulmonary KS	Pulmonary KS	3§	Neg	Pos	Death 23 wk after first IS
Pneumocystis colonization/ tuberculosis	Tuberculosis	3	Neg	Pos	Recovery
Pneumocystis colonization/ bacterial pneumonia	Bacterial pneumonia	2	Neg	Pos	Recovery
Pneumocystis colonization/ unspecified respiratory illness	Unspecified respiratory illness	2	Neg	Pos	Recovery
Pneumocystis colonization/ other diagnosis	Other diagnosis¶	1	Neg	Pos	Recovery
Unspecified respiratory illness	PcP	3	Neg	Neg	–
Tuberculosis	Tuberculosis	19	Neg	Neg	–
Bacterial pneumonia	Bacterial pneumonia	10	Neg	Neg	–
Unspecified respiratory illness	Unspecified respiratory illness	20	Neg	Neg	–
Unspecified respiratory illness	Unspecified respiratory illness	1	Neg	NA	Recovery (18 wk follow-up)
Other diagnoses¶	Other diagnoses¶	24	Neg	Neg	–
Other diagnosis¶	Other diagnosis¶	1	Neg	NA	Recovery (>1 y follow-up)

*IF, immunofluorescence stain; Pos, positive; Neg, negative; IS, induced sputum procedure; KS, Kaposi sarcoma; NA, not available.
†Diagnosis based on clinical evidence, IF, and PCR from an IS sample, and follow-up after episode.
‡Diagnosis based on clinical evidence and IF from an IS sample.
§Three episodes occurring in 1 person.
¶Among other diagnoses were sepsis, bronchitis, emphysema, pulmonary KS, and bronchiectasis.

Table 3. Incidence of *Pneumocystis* pneumonia (PcP) and other respiratory illnesses, Malawi, 2002–2004*

	All CD4 counts		CD4 0–99/mm^3		CD4 100–199/mm^3	
Diagnosis	No. events	Incidence, % (95% CI)	No. events	Incidence, % (95% CI)	No. events	Incidence, % (95% CI)
Confirmed PcP	6	1.0 (0.3–2.2)	5	5.7 (1.9–13.4)	1	0.6 (0.01–3.8)
Bacterial pneumonia†	102	17.3 (14.1–21.0)	35	40.2 (28.0–56.0)	42	28.6 (20.6–38.6)
Pulmonary tuberculosis	51	8.6 (6.4–11.3)	20	23.0 (14.0–35.5)	25	17.0 (11.0–25.1)
Unspecified respiratory illness	127	21.5 (17.9–25.6)	46	52.9 (38.7–70.5)	38	25.9 (18.3–35.5)

*CI, confidence interval.
†Diagnosis based on new consolidations shown on chest x–ray and response to antimicrobial drugs; includes patients with and without positive blood cultures.

with confirmed PcP cases (42.5 cells/mm^3, range 1–103) was not significantly lower than in those with *Pneumocystis* colonization (89.1 cells/mm^3, range 7–194; p = 0.28), but was significantly lower than in those with other diagnoses (97.0 cells/mm^3, range 1–311; p = 0.03). Mean age and sex distribution of confirmed PcP, *Pneumocystis* colonization, and other diagnoses were not significantly different. The case-fatality rate of confirmed PcP was 50%.

Conclusions

This is the first community-based prospective study of PcP in a developing country. We found an incidence of PcP in Malawian HIV-infected adults of 1.0/100 person-years, similar to the rates observed in studies that used less comprehensive diagnostic approaches in South African miners (0.5/100 person-years) (*11*) and the placebo arms of trials of trimethoprim-sulfamethoxazole prophylaxis in Côte d'Ivoire (*12*,*13*). The incidence in persons with CD4 counts <200/mm^3 (2.5/100 person-years) was clearly lower than in AIDS patients in the United States before the introduction of routine trimethoprim-sulfamethoxazole prophylaxis and highly active ART (10/100 person-years [*14*]). In the lowest CD4 count range (<100/mm^3), PcP was common, although the incidence was low compared with that of bacterial pneumonia and pulmonary tuberculosis.

We believe it is unlikely that we missed many PcP cases among other diagnoses or losses to follow-up because of the intensive active and passive follow-up and because our facility provided expeditious, high-quality care free of charge. Allowing for reduced sensitivity of induced sputum compared to BAL (*7*) and considering cases with diagnostic uncertainty as PcP cases would still leave the PcP incidence low in the HIV-infected population in general.

We found that *Pneumocystis* colonization and confirmed PcP were equally common among patients with suspected PcP. More sensitive molecular detection methods would possibly have detected higher rates of colonization. It remains uncertain why certain HIV-infected persons clear *Pneumocystis* colonization while others develop PcP. The level of immune suppression as indicated by the CD4 count is a possible explanation, although our data do not support this. Genetic differences between *P. jirovecii* strains may be relevant (*15*). Variation in worldwide distribution of strains, as well as differences in host genetics and shorter survival of patients in low CD4 count ranges, are possible causes of the lower PcP incidence in Africa than in developed countries.

The incidence of PcP in HIV-infected Malawian adults, diagnosed clinically and confirmed with molecular analysis, was low compared with the incidence of bacterial pneumonia and pulmonary tuberculosis at all levels of immunosuppression. PcP rarely occurred with CD4 cell counts >100 mm^3. Among the most immunocompromised patients, PcP is an important diagnostic consideration.

Dr van Oosterhout is a senior lecturer in the Department of Medicine, University of Malawi College of Medicine, Blantyre, Malawi. His research interests are the clinical aspects of HIV and the treatment of HIV and tuberculosis.

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Address for correspondence: Christopher V. Plowe, University of Maryland School of Medicine, 685 W Baltimore St, HSF1-480, Baltimore, MD 21201, USA; email: cplowe@medicine.umaryland.edu

Mycobacteria as Environmental Portent in Chesapeake Bay Fish Species

Andrew S. Kane,*†‡ Cynthia B. Stine,†‡ Laura Hungerford,*‡ Mark Matsche,§ Cindy Driscoll,§ and Ana M. Baya†¶

Infection with environmental mycobacteria is increasing among many Chesapeake Bay fish species. Prevalence in juvenile Atlantic menhaden differed between tributaries and ranged from 2% to 57%. Mycobacterial infection may be a syndromic sentinel of altered environmental conditions that threaten aquatic animal health.

An ongoing epizootic of mycobacteriosis has been reported among striped bass, *Morone saxatilis* (*1*), in the Chesapeake Bay, one of the largest and most productive estuaries in North America. Sampling and culture of striped bass from locations across the bay have led to the isolation of a number of distinct species of mycobacteria that occur alone or as polyinfections within individual fish (*2–4*). One contemporary approach to investigating such emerging infections is to use molecular techniques to focus on genetic characteristics and relatedness linking isolates (*5*). Alternatively, as with the opportunistic infections associated with human HIV, the key may not be solely the identity of the infecting bacteria. Rather, these pathogens may be a portent of more fundamental health disturbances that threaten multiple species within the Chesapeake Bay system. Laboratory studies suggest that other bay fish, such as Atlantic menhaden, are susceptible to multiple species of mycobacteria (*6*) similar to the variety of types isolated from infections in wild striped bass (*2–4*).

The purpose of our study was to survey a wider set of fish species, at multiple discrete locations within the Chesapeake Bay and its tributaries, for mycobacterial infection. Particularly, we examined juvenile Atlantic menhaden because they are a "keystone" species in the bay. Ecologically, Atlantic menhaden represent the highest level (taxonomically) filter feeder in the bay. This may be of notable consequence because menhaden filter enormous volumes of sediment and plankton to derive nutrition (*7*), and aquatic mycobacteria have an affinity for growing on particles, biofilms and sediments, and to be incorporated into amoebae, algae, and other microorganisms (*8*,*9*). Furthermore, menhaden provide a critical forage base for other animals and support "recruitment" to the bay's adult fisheries.

*University of Maryland School of Medicine, Baltimore, Maryland, USA; †Virginia-Maryland Regional College of Veterinary Medicine, College Park, Maryland, USA; ‡University System of Maryland, College Park, Maryland, USA; §Maryland Department of Natural Resources, Oxford, Maryland, USA; and ¶Maryland Department of Agriculture, College Park, Maryland, USA

The Study

Fish were collected by beach seine, cast net, or bank trap from the Choptank, Chicamacomico, Nanticoke, and Pocomoke Rivers of the Chesapeake Bay. Live fish were transported in oxygenated, insulated coolers to the University of Maryland Aquatic Pathobiology Center for examination and microbiology. Liver (Atlantic menhaden) and spleen (other fish species) tissues were sampled aseptically, homogenized in Butterfield's phosphate-buffered saline and plated on Middlebrook 7H10 agar (Difco, Detroit, MI, USA) supplemented with Bacto Middlebrook oleic acid, albumin, dextrose, catalase (Difco). Plates were assessed for colony growth after 2–8 weeks, and mycobacteria were identified to the genus level on the basis of colony morphology, growth characteristics, and gas chromatographic fatty acid methyl ester analyses. Prevalence of mycobacterial infection (proportion of sampled fish with positive culture results in each subgroup) was calculated for each species. Prevalence in juvenile Atlantic menhaden was compared among the river systems by using the Fisher exact test.

Mycobacteria were recovered from Atlantic menhaden, white perch, blueback herring, largemouth bass, mummichog, striped killifish, summer flounder, weakfish, and spot (Table 1). No externally visible lesions were present on fish of any species sampled, except Atlantic menhaden. A low percentage (<10%) of the sampled Atlantic menhaden had visible signs of disease, mainly external, often perianal ulcers penetrating through the skin and the underlying musculature. Histologic results from a subsample of these fish indicated that these lesions were consistent with ulcerative mycosis.

Prevalence of mycobacterial infection among wild-caught, juvenile Atlantic menhaden ranged from 2% to 57% by river system (Table 2). Atlantic menhaden from the Chicamacomico River had a notably higher prevalence of infection ($p<0.001$) than menhaden sampled from the other river systems.

Conclusions

Mycobacteriosis in the Chesapeake Bay is a problem of much wider scope than the previously recognized epizootic in striped bass (*1*). In this report, we document

Table 1. Prevalence of culture-confirmed mycobacteriosis among fish sampled from mid–Chesapeake Bay tributaries

Species (n)	% Culture-positive	95% Confidence interval
Atlantic menhaden (287) *Brevoortia tyrannus*	18	13.5–22.7
Blueback herring (17) *Alosa aestivalis*	12	1.5–36.4
Summer flounder (26) *Paralichthyes dentatus*	12	2.5–30.2
Striped killifish (1) *Fundulus majalis*	100	2.5–100.0
Mummichog (3) *F. heteroclitus*	33	0.8–90.6
Largemouth bass (1) *Micropterus salmoides*	100	2.5–100.0
Weakfish (2) *Cynoscion regalis*	50	1.3–98.7
Spot (27) *Leiostomus xanthurus*	7	0.9–24.3
White perch (87) *Morone americana*	20	11.8–29.4

infection in multiple fish species representing a range of life histories, water strata, and locations. Why mycobacteriosis has emerged in this setting is unclear: the new findings reshape future investigations from a single host–single pathogen focus to consideration of the ecology of multiple hosts and related pathogens within a dynamic system. Certain water quality criteria, including those associated with degraded habitats, lower pH, and higher organic content, have been reported to foster the growth of environmental mycobacteria (*9*). Other factors, such as increases in suspended particulates, biofilms, and even water dynamics associated with global warming, may support enhanced growth of environmental pathogens including mycobacteria (*9*,*10*). Metal and organic contamination, algal blooms, and low dissolved oxygen levels serve as environmental stressors in the Chesapeake Bay (*11*). Water quality has strong spatial heterogeneity and temporal flux, and these conditions could exacerbate both bacterial proliferation and host susceptibility.

Variability in prevalence across species and locations provides opportunities to determine the underlying ecology of emerging infections. Preliminary water quality data from 3 of the 4 Atlantic menhaden sampling locations at the time of fish collection show that pH was lower in the Chicamacomico (6.9, standard deviation [SD] ± 0.3) and Pocomoke Rivers (7.1, SD ± 0.3), where mycobacterial prevalence was higher, than in the Choptank River (8.3, SD ± 0.4), where prevalence was lower. Further, dissolved organic carbon was notably higher in the Chicamacomico (16.5 mg/L, SD ± 1.1) and Pocomoke Rivers (11.7 mg/L, SD ± 0.5) than in the Choptank River (4.4, SD ± 0.4).

The prevalence of infection in Atlantic menhaden is notable and may indicate the potential of this fish to amplify spread to other species, as they are an essential link in the food chain. The Atlantic menhaden fishery, the largest commercial fishery in the Chesapeake Bay, provides an important source of protein (as fish meal) in animal feeds for both agricultural and domestic pets, as well as oils rich in omega-3 fatty acids used in human and veterinary diet supplements, and as bases for cosmetics.

Aquatic mycobacteria cause opportunistic infections and disease in humans, most commonly among those who are immunocompromised or have other serious diseases, or following a skin abrasion or penetrating wound (*12*,*13*). The prevalence of mycobacterial infection in this study raises concern for potential increases in human infections in the Chesapeake Bay region through contact with fish, as well as through recreational contact and drinking water (*8*,*9*,*13*–*15*).

Mycobacterial infections in bay fish may serve as a syndromic sentinel of an environmental state that already affects the health of multiple inhabitants of the region. Data from this and ongoing studies refocus attention on the complex, anthropogenically accelerated changes that may be altering the distribution of emerging diseases worldwide. The Chesapeake Bay is an important ecosystem with a large urban component, so understanding the epidemiology of this multispecies epizootic may improve not only the health of local inhabitants but also the prediction of other emerging infections.

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Diagnostic and handling procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland, College Park, Maryland. Portions of this study were supported through the National Oceanic and Atmospheric

Table 2. Prevalence of culture-confirmed mycobacteriosis among Atlantic menhaden from 4 Chesapeake Bay tributaries

River (n)	% Culture-positive*	95% Confidence interval
Nanticoke (60)	2[a]	0.04–8.9
Choptank (134)	10[a,b]	5.3–16.0
Pocomoke (46)	21[b]	11.0–36.4
Chicamacomico (47)	57[c]	42.2–71.7

*Small letters a, b, and c indicate a significant difference from other river systems at p<0.01 (Bonferroni-corrected Fisher exact test probabilities).

Administration, Chesapeake Bay Office, the Maryland Department of Health and Mental Hygiene, and Centers for Disease Control and Prevention.

Dr Kane is associate professor in the Department of Epidemiology and Preventive Medicine, University of Maryland School of Medicine, Baltimore, Maryland. His research focuses on environmental pathology, infectious diseases, and toxicology of aquatic organisms, with emphasis on the Chesapeake Bay and its fauna.

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Address for correspondence: Andrew S. Kane, Aquatic Pathobiology Center, University of Maryland, 8075 Greenmead Dr, College Park, MD 20742, USA; email: akane@umaryland.edu

Yersinia pestis Orientalis in Remains of Ancient Plague Patients

Michel Drancourt,* Michel Signoli,* La Vu Dang,* Bruno Bizot,*† Véronique Roux,* Stéfan Tzortzis,*‡ and Didier Raoult*

Yersinia pestis DNA was recently detected in human remains from 2 ancient plague pandemics in France and Germany. We have now sequenced *Y. pestis glp*D gene in such remains, showing a 93-bp deletion specific for biotype Orientalis. These data show that only Orientalis type caused the 3 plague pandemics.

Three historical pandemics have been attributed to plague. The causative agent, *Yersinia pestis*, was discovered at the beginning of the ongoing third pandemic. The etiology of the 5th-7th–century first pandemic and the 14th-18th–century second pandemic, however, remained putative until recently (*1*). Indeed, results of 16S rRNA gene-based detection using teeth collected from 64 persons' remains in 7 northern Europe sites remained negative (*2*). When using different molecular targets and the dental pulp as a suitable specimen, we detected *Y. pestis*–specific DNA fragments in European skeletons of persons suspected of having historical plague (*3*–*5*). Our results were independently confirmed on 6th-century Bavarian teeth (*6*). *Y. pestis* comprises biotypes Antiqua, Medievalis, and Orientalis, recognized on the basis of the conversion of nitrate to nitrite and fermentation of glycerol. A fourth biotype, Microtus, describes Medievalis isolates lacking arabinose fermentation. In 1951, Devignat proposed that each of the first 3 biotypes determined each plague pandemic (*7*). This hypothesis was challenged by our multispacer-typing detection of an Orientalis-like biotype in 5th- to 14th-century dental pulp specimens (*5*). A 93-bp deletion from the *Y. pestis glp*D gene encoding the glycerol-3-phosphate dehydrogenase determines lack of glycerol fermentation of the Orientalis biotype (*8*,*9*). Isolates of the other biotypes lack this deletion (*8*). Here, we establish role of Orientalis biotype in the 3 pandemics by sequencing the *glp*D gene from additional ancient dental pulp specimens.

*Université de la Méditerranée, Marseille, France; †Ministère de la Culture, Paris, France; and ‡Service archéologique, Martigues, France

The Study

We had historical evidence that 3 mass graves excavated in France were used to bury bubonic plague victims. In Vienne, 12 skeletons, including 5 children, buried within the ruins of a Roman temple have been dated from the 7th–9th centuries both by a 5th-century coin and ^{14}C dating. In Martigues, 205 skeletons buried in 5 trenches were dated from 1720 to 1721 on the basis of coins and detailed parish bills that listed the victims (Figure). In Marseille, 216 skeletons buried in a huge pit dated from a May 1722 epidemic relapse. We previously confirmed the diagnosis of plague at this site (*3*). Eighteen teeth from 5 skeletons in Vienne, 13 teeth from 5 skeletons in Martigues, and 5 teeth from 3 skeletons in Marseille were processed for the search for *Y. pestis* DNA in the dental pulp. The teeth were processed according to published criteria for authenticating molecular data in paleomicrobiology (*10*): 1) there should be no positive control; 2) negative controls, as similar as possible to the ancient specimens, should test negative; 3) a new primer sequence targeting a genome region not previously amplified in the laboratory should be used (suicide PCR); 4) any amplicon should be sequenced; 5) a second amplified and sequenced target should confirm any positive result; and 6) an original sequence that differs from modern homologs should be obtained to exclude contamination.

Accordingly, DNA samples were submitted for suicide-nested PCR conducted by using 1 negative control (18th-century teeth from skeletons of persons without anthropologic and macroscopic evidence of infection) for every 3 specimens. Two microliters (1 μL for nested PCR) DNA were amplified in a 50-μL mixture containing 10 pmol of each primer, 200 μmol/L each deoxyribonucleotide triphosphate (Invitrogen, Cergy-Pontoise, France), 1.5 U *Taq* polymerase (Invitrogen), and 2.5 μL of a 50-mmol/L solution of $MgCl_2$ in 1× *Taq* buffer. Nested PCR aimed to encompass the entire *glp*D gene incorporated primers: glpD-F1: 5′-GGC TAG CCG CCT CAA CAA

Figure. Skeletons from a mass grave in Martigues, 1720–1721, yielded molecular evidence for the *Yersinia pestis* Orientalis biotype. Photograph: S. Tzortzis.

AAA CAT-3′ (positions 170080–170103, reference: *Y. pestis* strain CO92 genome sequence AJ414159.1)/glpD-R2: 5′-GGT GCC AGT TTC AGT AAC AC-3′ (positions 170402–170383) for initial PCR and glpD-F3: 5′-CGC TGT TTC GAA CAT TCA GA-3′ (positions 170230–170249) /glpD-R3: 5′-GGC CAA GGC TTC ACT TAC CA-3′ (positions 170373–170354) for nested PCR. PCRs were performed in a T3 thermocycler (Biolabo, Archamps, France) under the following conditions: an initial 2 min of denaturation at 95°C was followed by 43 cycles (38 cycles for nested PCR) of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 90 s at 68°C. The amplification was completed by holding the reaction mixture for 7 min at 68°C. PCR products purified by using a MultiScreen PCR plate (Millipore Corp., Bedford, MA, USA) were sequenced with a DNA sequencing kit (Big Dye Terminator Cycle Sequencing V2.0; PE Biosystem, Courtaboeuf, France) and subjected to electrophoresis with the 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were compared in the GenBank database (www.ncbi.nlm.nih.gov/GenBank) using the multisequence alignment Clustal within the BISANCE environment.

No amplification was observed in 11 negative controls, but 5 of 36 teeth yielded an amplicon of 191-bp length in 2 of 4 persons' remains from Vienne, 2 of 5 from Martigues, and 1 of 3 from Marseille. Amplicons exhibited 100% sequence similarity with that of the *Y. pestis* Orientalis *glp*D gene (GenBank accession nos. AY312359 for tooth 35–0235, Vienne; DQ073797 for tooth SQ401521 and DQ073798 for tooth SQ408113, Martigues; and AY312360 for tooth 25–0225, Marseille) and were characterized by a 93-bp deletion when compared with the *glp*D gene sequence of the *Y. pestis* Medievalis biotype (GenBank accession no. AE 013994).

Conclusions

In this study, contamination of the ancient specimens is unlikely because of the extensive precautions we took, including use of the suicide PCR protocol excluding positive controls (*4*). Accordingly, *glp*D gene had never been investigated in our laboratory before this study, and negative controls remained negative. The specificity of the amplicons was ensured by complete similarity of experimental sequences with that of the *Y. pestis* Orientalis *glp*D gene (*8*). One site (Marseille, 1722) was previously positive for *Y. pestis* after sequencing of 2 different targets (chromosome-borne *rpob* and plasmid-borne *pla* genes) in other specimens collected in other persons' remains (*3*).

These results therefore confirm the detection of *Y. pestis*–specific DNA in plague patients' remains from the first and second epidemics (*3–6*). We observed a 93-bp in-frame deletion within the *glp*D gene sequences obtained from ancient dental pulp specimens. This deletion has been found only in Orientalis biotype isolates in 2 independent studies comprising a total of 77 and 260 *Y. pestis* isolates, respectively, of the 4 biotypes (*8,9*).

After previous demonstration of *Y. pestis* Orientalis-type multiple spacer type sequences in Justinian and medieval specimens (*5*), we now have cumulative evidence using 2 different molecular approaches that *Y. pestis* closely related to the Orientalis biotype was responsible for the 3 historical plague pandemics.

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Mr Drancourt is professor of medical microbiology in Unité des Rickettsies, Marseille Médical School, Marseille, France. His research interests are paleomicrobiology of plague and bartonelloses.

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Address for correspondence: Didier Raoult, Unité des Rickettsies CNRS UMR 6020, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseille CEDEX 05, France; email: didier.raoult@medecine.univ-mrs.fr

Rickettsia parkeri Infection after Tick Bite, Virginia

Timothy J. Whitman,* Allen L. Richards,† Christopher D. Paddock,‡ Cindy L. Tamminga,† Patrick J. Sniezek,* Ju Jiang,† David K. Byers,* and John W. Sanders*

We describe a man with a febrile illness and an eschar that developed at the site of a tick bite. *Rickettsia parkeri* was detected and isolated from the eschar. This report represents the second documented case of *R. parkeri* rickettsiosis in a US serviceman in eastern Virginia.

In the United States, 4 species of spotted fever group (SFG) rickettsiae are recognized as pathogens of humans. These include *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever (RMSF); *R. felis*, the cause of fleaborne spotted fever; *R. akari*, the agent of rickettsialpox; and *R. parkeri* (*1*,*2*). Of these, *R. rickettsii* is the only pathogen definitely associated with tick bites.

In 2004, Paddock et al. described the first recognized case of infection in a patient with *R. parkeri (1).* That patient, a US serviceman living in the Tidewater region of eastern Virginia, had a mild febrile illness and multiple eschars. He reported frequent tick and flea exposures but could not recall a specific arthropod bite in the month before illness. However, *R. parkeri*, a tick-associated rickettsia (*3*), was subsequently isolated in cell culture from 1 eschar. (*1*) We present the second known case of spotted fever due to *R. parkeri* in a serviceman (the third case overall) and its unequivocal association with tick bite.

The Case

A 53-year-old US serviceman was seen at our clinic on September 8, 2006; he reported a 2-day history of fever, malaise, and rash. He denied headache, nausea, vomiting, or myalgia. He had recently returned from a vacation in the Virginia Beach area, where he had removed a large brown tick with white markings from his right pretibial region. The patient estimated that the tick had been attached ≈8 hours before being removed. Four days after the tick was removed, an eschar developed at the bite site. Three days later, temperatures up to 39°C and drenching night sweats that persisted for 2 days developed. He then sought care.

*National Naval Medical Center, Bethesda, Maryland, USA; †Naval Medical Research Center, Silver Spring, Maryland, USA; and ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA

On examination, his temperature was 38°C. A nontender, nonpruritic eschar that measured 1 cm × 1 cm was visible on his right lower extremity (Figure 1) along with ≈15 nontender, nonpruritic papules on his torso, upper arms, and legs (Figure 2). The remainder of his physical examination was normal. Laboratory studies were normal except for a leukocyte count of 3.4×10^9 cells/L (normal $4–11 \times 10^9$ cells/L) and an aspartate aminotransferase level of 38 U/L (normal 8–33 U/L).

The patient was admitted to the hospital and treated with oral doxycycline 100 mg twice a day. Fevers and malaise immediately resolved after the first dose. The patient was discharged, and the rash resolved after 3–4 days of therapy.

DNA extracted from skin-biopsy specimens of the proximal tibial eschar and a shoulder papule was positive by 2 real-time PCR assays designed to amplify segments of the 17-kDa antigen and outer membrane protein B genes of all *Rickettsia* spp. and tickborne SFG rickettsiae, respectively *(4,5)*. Amplicons obtained from 2 additional genes, *gltA* (333 bp) and *sca4* (849 bp), were sequenced and determined to be 100% identical to those of *R. parkeri* (GenInfo Identifier nos. 1389996; 13568656). The histopathology of the shoulder papule showed mild superficial perivascular inflammation without eosinophils. Acute-phase serum and whole blood samples from the patient were also tested by real-time PCR and were negative. *R. parkeri* (Ft. Story strain) was isolated in Vero E6 cells from a portion of the eschar biopsy specimen. Immunohistochemical staining of the shoulder papule identified occasional spotted fever rickettsiae in the inflammatory cell infiltrate. Acute- and convalescent-phase serum specimens were tested for immunoglobulin G antibodies reactive with SFG and typhus group (TG) rickettsiae by using ELISAs with *R. rickettsii* and *R. typhi* antigens. The titers for the SFG ELISA acute- and convalescent-phase serum samples were <100 and 1,600,

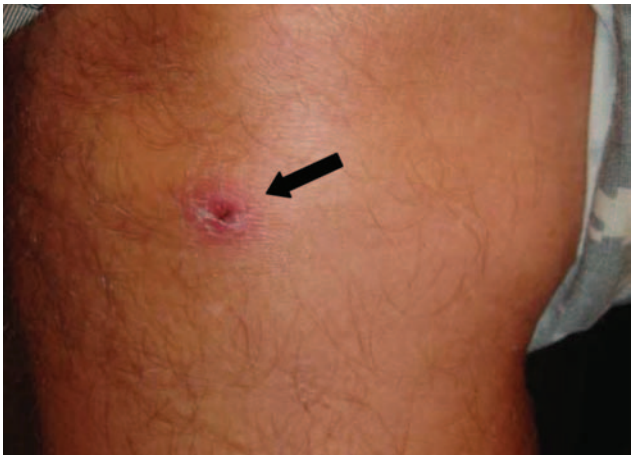

Figure 1. Eschar on right pretibial region (arrow).

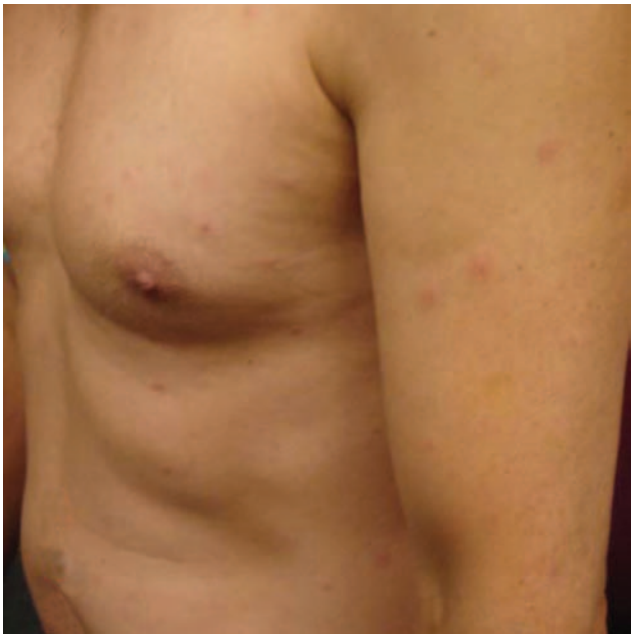

Figure 2. Multiple papules on torso and upper arms.

respectively. Both samples were negative (titer <100) for the TG ELISA.

Conclusions

R. parkeri was first isolated in 1937 from *Amyblyomma maculatum* ticks found on cattle in the Gulf Coast region of Texas (*3*), but its role as a human pathogen was unknown. For many years investigators speculated that agents other than *R. rickettsii*, including *R. parkeri*, caused mild RMSF-like illnesses in the United States (*6*,*7*). However, the role of *R. parkeri* as a pathogen of humans was not confirmed until 2002, when *R. parkeri* was isolated from a patient with a relatively mild febrile illness and multiple eschars (*1*). Confirmed cases of *R. parkeri* rickettsiosis have been described only twice (*1*,*8*), and the prevalence of this disease is unknown; however, many cases of this infection have likely been misidentified as RMSF (*6*). Recently, Raoult and Paddock analyzed serum specimens of 15 US patients who had an earlier diagnosis of RMSF and identified 4 that reacted with a 120-kDa protein of *R. parkeri*, a finding suggestive of infection with this agent (*9*). This hypothesis is further supported by seroprevalence studies of US military personnel that showed that 10% of those tested were positive for SFG rickettsiae when a *R. rickettsii* ELISA antigen was used (*10*). Because of recognized cross-reactivity of *R. rickettsii* with antigens of other SFG rickettsiae, including *R. parkeri*, this level of serologic reactivity could represent mild, self-limited infections with other SFG rickettsiae, including *R. parkeri*, that are less virulent than *R. rickettsii*.

Our patient had a single eschar at the site of tick attachment. Although it has been presumed that ticks are involved in the transmission of this disease, this is the first clear documentation of this occurrence. The infection can apparently be transmitted within several hours of attachment because the patient was certain of the maximum interval that the tick could have been attached to a visible region of his leg. Finally, the general description of the tick matches that of *A. maculatum*, the putative vector of *R. parkeri* rickettsiosis, and this tick has been collected previously in southeastern Virginia (*11*).

R. parkeri is the newest member of the SFG rickettsiae in the Western Hemisphere to be conclusively associated with illness in humans (*12*). Because 2 of the 3 patients described to date were US servicemen in the Virginia Tidewater region, where thousands of military personnel are stationed, this tickborne infection has potential military relevance. Serologic tests specifically for *R. parkeri* are not widely available, and an accurate diagnosis is best made by using PCR testing of a biopsy specimen of eschars or papules. A definitive diagnosis can also be made by using cell culture isolation techniques with the same biopsy specimen, as described for other eschar-associated rickettsioses (*12*).

Clinicians should be aware of this newly noted rickettsial illness, specifically in the eastern coastal region of the United States. They should consider this diagnosis for patients with mild rickettsiosis-like illness and single or multiple eschars.

Dr Whitman is an infectious disease specialist at the National Naval Medical Center and an assistant professor of medicine at the Uniformed Services University in Bethesda, Maryland, USA. His research is focused on community-associated methicillin-resistant *Staphylococcus aureus*.

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Address for correspondence: LCDR Timothy J. Whitman, US Navy Infectious Diseases Department, National Naval Medical Center, Bethesda, MD 20889 USA; email: tjwhitman@bethesda.med.navy.mil

Scorpion found within the base camp hospital. Photo by R.T. Foster Sr.

Mal de Mayo

Raymond T. Foster, Sr.*†

A few miles from the Honduran border, we waited for the closing ceremonies to mark the conclusion of our humanitarian efforts. Despite the oppressive, unrelenting heat, stifling body odor, the poverty surrounding us, and our longing to be reunited with family, the soldiers and airmen of Combined Joint Task Force Bravo were extremely proud of their accomplishments after months of being in El Salvador. This task force had been deployed to El Salvador as part of Operation New Horizons 2006, which is undertaken every year at multiple locations in Central and South America. As an Army physician, I had been assigned to be the task force surgeon.

The mountains of the northern Morazán region had been devastated by 14 years of civil war. But thanks to our task force, at least in part, this devastation was now softened with new schools and clinics in some of the most inaccessible areas of this nation. The backdrop for today's celebration was a newly christened clinic devoted to gynecologic and maternal health. It seems I had left my comfortable professional environment at Duke University and traveled 1,700 miles to help construct clinics similar to those to which my civilian career was dedicated.

After the US ambassador's remarks, I would enjoy attempts to communicate with some of the local physicians and nurses who had attended the ceremony. Despite the language barrier, we were able to communicate at a level that superseded our mutual difficulties. The happiness and hope of these professionals, of the local families present, and of the scores of uniformed school children who had been escorted there for the festivities validated our hardship and sacrifice. My thoughts turned back to the past several weeks.

I had arrived on May 17, 2006, coincident with the beginning of Central America's rainy season. The ride from San Salvador to Camp Morazán took 3½ hours and included an afternoon torrential rainstorm followed quickly by temperatures in excess of 110°F. Our engine overheated, and we were briefly stranded alongside the only hard surface road in that region.

Upon arrival at our base camp, I reported to the Sergeants Major on duty and quickly discovered that urogynecology is not the preferred medical specialty for con-

*Duke University Medical Center, Durham, North Carolina, USA; and †320th Combat Support Hospital, Greensboro, North Carolina, USA

struction accidents and tropical illness. Despite the uncomfortable conversation, I wanted the encounter to last indefinitely because the portable military air-conditioning unit attached to the headquarters tent made my temporary surroundings a pleasant, almost chilly, 90ºF.

I found the hospital tent in the dark by following the sound of the generators powering lights and essential medical equipment inside the small network of tents that formed an impromptu hospital in an El Salvadorian cow pasture. I quickly regretted that the new 80-hour workweek restrictions for my residents at Duke do not apply to Army physicians.

During my first evening in the camp hospital tent, I attended to patients with minor trauma, scorpion stings, spider bites, and the work of the insect known affectionately as the "pissing beetle." This insect, which I thankfully never personally experienced, was drawn to the neck and face of sleeping soldiers. A secreted liquid left an irritating and unattractive rash (Figure). The local remedy, juice squeezed from freshly cut limes directly onto the skin, was quite effective, and it left the otherwise pungent patients with a fresh citrus scent.

Adjacent to our base camp, a small, unkempt dwelling without electricity or running water housed a half-dozen adults and children. They shared their sparse dwelling with scores of chickens and several roosters. The fowl quickly had learned how to move through the razor wire that lined the perimeter of our camp. Thanks to these wonderful animals, I never missed an opportunity to awaken at 4:30 AM, which left me ample time to prepare for breakfast in the mess tent at 5:30 AM each day.

After breakfast on my first full day, I attended to patients at morning "sick call" and resumed the task of familiarizing myself with our pharmacy supplies, emergency medical equipment, evacuation procedures, and our medical documentation and charting process. At that time, it appeared as though the Army had well prepared me for various scenarios that might jeopardize the health of the more than 500 American and Salvadoran soldiers whose care was my responsibility. However, my optimism would shortly crumble.

On my third day in country, Combined Joint Task Force Bravo, which had been operating for nearly 5 months, began planning and preparation for redeployment of more than 250 military transport and heavy construction vehicles to the seaport. This meant that my hospital facilities would be disassembled and packed into shipping containers over the next 48 hours. I was to practice "tailgate medicine" from the back of 2 army ambulances, which would not be sent on the 5-hour journey to the seaport until the last convoy. I selected a small supply of medicines that I imagined would be useful for my impromptu clinic. With the ever-present stifling heat, I moved many cases of intravenous fluid into the ambulances to use for the steady flow of daily heat casualties. I armed my wheeled clinic with only 2 small bottles of antidiarrheal tablets (loperamide) and a single container of antiemetic medication (metoclopramide). I had not yet seen any gastrointestinal illness and did not anticipate that it would become a problem.

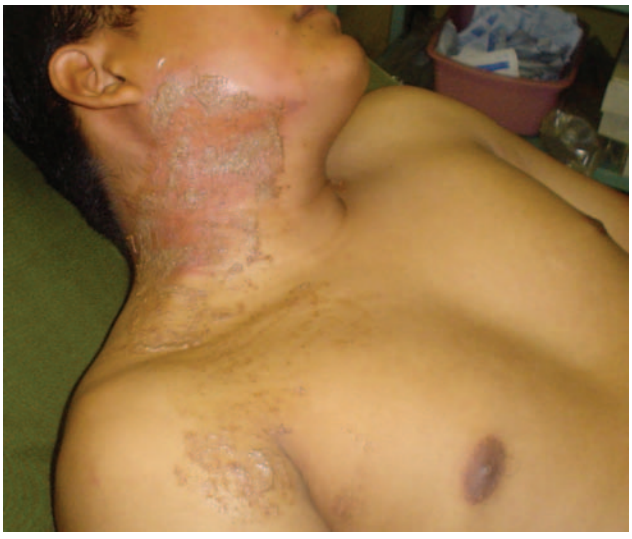

Figure. El Salvadorian infantry soldier (commando) with the characteristic rash seen after an encounter with the "pissing beetle" while sleeping. Photo by R.T. Foster Sr.

The heat, interspersed with impressive thunderstorms, continued. The day that the shipping containers packed with my mobile hospital departed for the seaport on flatbed trucks, I a saw small number of patients with acute, severe gastrointestinal symptoms. These soldiers consistently complained of copious, nearly explosive diarrhea. Other symptoms included myalgia, headache, fever, nausea, and vomiting. The frequent diarrhea, described by most soldiers as 10 or more bowel movements per day, led to dehydration for most, which was compounded by the fluid loss from the extreme heat.

To further exacerbate everyone's misery, the plastic, portable latrines stationed around our base camp often reached interior temperatures in excess of 120ºF. Some soldiers were spending up to 2 hours per day sitting in an oven, perched above gallons of foul-smelling diarrhea. As illness grew throughout the task force, I met with our task force commander, Colonel William Buckler.

The commander already knew that key members of his staff were operating at reduced efficiency. He asked me to assess the problem so that any resources we needed from our host nation could be requested. Without medical documentation, I could only estimate that nearly half of our task force had symptomatic illness, some with severe symptoms and dehydration. Colonel Buckler became concerned not only for the welfare of soldiers under his com-

mand, both US forces and Salvadoran military personnel, but also for this disease's effect on our ability to finish construction at remote sites near the Honduran border.

The task force staff included an Army counterintelligence agent, whom we'll call Mr. Barreda. The mission of Mr. Barreda was to assimilate himself into the local population and notice any change in the political or social situation that might endanger our command. I was given the opportunity to meet with this crafty individual and assign him specific questions for which to seek answers during his normal interactions with the locals. Our greatest threat, however, was now identified as liquid, brown, malodorous, and ever-present in more copious amounts.

Meanwhile, assuming that patients had viral gastroenteritis, I instituted strict handwashing at meal time and in the latrine areas. I met with our cheerful yet completely overworked cook, Staff Sergeant Howard. With the help of locally hired labor, he supervised preparation of 2 hot meals, which were usually the pinnacle of our day. Sergeant Howard queried each of the food service workers about any gastrointestinal symptoms. With the assistance of a bilingual member of our command, he completed these interviews in short order. The workers reported no symptoms; however, because these local laborers feared loss of income should they be known to have a communicable disease, they may not have been completely forthcoming.

My stomach, as well as my brain, was churning as I continued to struggle with this gastrointestinal puzzle. My suspicions of a common gastroenteritis virus (such as Norwalk virus) were lessened as I noted a persistence of symptoms, especially among those with the most severe symptoms, which lingered from days to weeks. Some patients even remained ill for more than a month. My symptoms waxed and waned, consistent with constant exposure and reinfection. A confounding variable was the gastrointestinal symptoms of soldiers who were taking weekly malaria prophylaxis, which often caused similar, although less severe, digestive symptoms. In fact, many patients had already had symptoms, coincident with beginning chloroquine therapy, before even arriving in Central America.

Without access to any diagnostic aids, such as simple blood chemistries, microbiologic studies, or imaging, I might never know the exact cause of this now-rampant illness. To assess the effect on our combat strength, I instituted a diarrhea checkpoint in the chow line. Army medics asked every soldier in his or her own language, while the soldier waited to enter the food service line, if he or she had symptoms of diarrhea, nausea, or vomiting. In addition to our newly established checkpoint, I used my flashlight for daily latrine inspections. Timing inspections just before the arrival of the sanitation trucks that emptied the latrines, I was able to get a sense of how much formed stool versus liquid stool was being passed by our soldiers in each 24-hour period.

Most unexpectedly, Mr. Barreda reappeared. We had a brief conversation, and he handed me some handwritten notes, which introduced me to the phrase "mal de Mayo" (strictly translated "bad of May" and more commonly used to mean "illness of May"). Mal de Mayo was an annual event that included symptoms consistent with those of our soldiers. Mal de Mayo was associated with the rainy season, which brought countless varieties of flying insects that were presumed vectors for this poorly characterized disease. The local population was not overly concerned beyond seeking parenteral rehydration for the very young, very old, and the most severely affected. Mr. Barreda also handed me a ragged piece of paper with a short list of local remedies (in Spanish) for mal de Mayo: "cloranfenicol, yodoclorina, alka AD, intestonomicina, oreganito."

Beyond mal de Mayo, Mr. Barreda informed me that several years earlier, Central America had experienced a rotavirus epidemic, which had caused unprecedented illness and death. Since the outbreak, the fragile El Salvadorian medical infrastructure was overwhelmed each May as patients sought reassurance that family members would not succumb to a potentially deadly infection.

My gynecologic training had not prepared me for rotavirus, other than it was a viral bowel infection particularly worrisome in newborns. I had vague recollections of *Giardia* and *Cryptosporidia*, but I was not able to recall any specific signs and symptoms beyond generic gastrointestinal complaints. My diarrhea checkpoint and daily latrine inspections led me to believe that about 60% of our forces had symptomatic illness.

Soldiers from both countries left the base camp each morning in route to construction sites. Other soldiers continued to work toward preparing and moving equipment to the port.

As an avid reader of military history, I thought often of the similarities between our mission in El Salvador and how horrific it must have been to construct the Panama Canal while battling yellow fever. History recorded that in 1884, Ferdinand de Lesseps took 500 young French engineers to Panama to supervise the construction project that he predicted would last 3 years. None of these 500 professionals lived to receive their first month's pay. Despite this catastrophic setback, de Lesseps persisted until he lost more than 30% of his workforce consisting of 20,000 Europeans (*1*). In my estimation, Colonel William C. Gorgas (for whom the US Army hospital in Panama City is named) is the true reason the canal exists. His understanding of disease transmission, mostly gathered from the work of Major Walter Reed, led him to aggressively rid the canal construction areas of the *Aedes aegypti* mosquito (*2*). His efforts almost eliminated yellow fever from

the workforce and fostered completion of the canal in 1904.

Unlike Colonel Gorgas' success with illness plaguing the Panama Canal construction, I had no idea what might be causing disease in our population. Furthermore, aggressive field hygiene techniques, including fog treatments to rid our base camp of mosquitoes and other flying potential disease vectors, had failed to lessen the illness. All we could do was continue providing supportive care to affected soldiers. Loperamide did not usually help, and metoclopramide often increased the severity of the symptoms. Intravenous fluids and antipyretics were the mainstay of therapy. We did our best to protect ill soldiers from the heat and allow them as much rest as possible. We balanced the need for patient convalescence with our mandate for mission completion. We survived day to day, and we were all inspired by the selfless attitudes displayed by our patients. Days and weeks passed, and despite the overwhelming disease burden, the mission neared completion and task force staff shifted efforts to the planning and execution of closing ceremonies.

Within 2 days of the closing ceremonies, the base camp was disassembled beyond the point of meeting minimal requirements for soldier support. I remained in San Miguel with 4 medics and a single ambulance. In the hotel, we typically saw patients morning and night in their rooms, in effect conducting patient rounds. We continued to concentrate on supportive care, still unaware of the exact cause of the illness affecting so many members of our unit.

Twenty-two days after arriving in El Salvador, I boarded my return flight as one of the last members of the command to depart the theater of operations. During my brief tenure as task force surgeon, I became a better doctor. I never determined what causes mal de Mayo. I cannot even imagine why it spread so rapidly and affected so many people. I did, however, refocus myself on basic skills of medical practice. I took detailed histories and did countless physical exams, upon which my treatment decisions were made. I had no expensive tests to guide my decisions. I often focused much effort on simply comforting those whose symptoms were unrelenting.

At the writing of this story, I am unaware that any family members or cohabitants of soldiers who arrived home with persistent symptoms have become ill. My curiosity led me to ask questions and search the medical literature upon return from Central America. I was quickly dissatisfied with what I was able to learn from Medline investigation, internet searches, and informal conversations with colleagues trained in infectious disease. According to the Cultural Profiles Project, funded by Citizenship and Immigration Canada, Central America's rainy season forces much of Central America's improperly managed sewage into the water supply, leading to "dysentery and diarrhea"(*3*). I also found evidence to suggest that *Cyclospora* might be the responsible organism in contaminated drinking water in Central America (*4*). However, our soldiers were forbidden to drink from the local water supply. Bottled water and water purified by a military reverse osmosis water purification unit, tested and approved by the US Army preventive medicine team attached to the task force, was consistently available at the base camp and all construction sites.

The challenge of future US humanitarian missions in Central America will be to anticipate and care for persons affected by mal de Mayo. I am encouraged that although this disease affected the gastrointestinal tract, it missed the heart of the soldiers and airmen assigned to Combined Joint Task Force Bravo.

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Dr Foster is a third-year fellow in urogynecology and pelvic reconstructive surgery at Duke University in Durham, North Carolina, and a member of the 320th Combat Support Hospital in Greensboro. His primary research interests include using a nonhuman primate (squirrel monkey) model to study the molecular and cellular basis of pelvic organ prolapse.

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Address for correspondence: Raymond T. Foster, Sr., Box 3192, DUMC Durham, NC 27710, USA; email: raymond.foster@duke.edu

Compensation for Avian Influenza Cleanup

To the Editor: Since 2003, highly pathogenic avian influenza (HPAI) H5N1 has shaken the world. In 10 countries, 258 confirmed cases in humans and 154 deaths have been reported (*1*). The number of countries with confirmed HPAI in poultry and wild birds jumps to 54 (*2*). Almost all persons infected with H5N1 have had close contact with sick or dead poultry by having butchered them, plucked them, or played (children) with them (*3*). Because H5N1 can potentially mutate or reassort into a strain capable of efficient human-to-human transmission, rapid elimination of the H5N1 virus in poultry and other risk-reduction interventions are thought to be essential for preventing further spread of HPAI (*4*). As a result, thousands of workers around the world have culled millions of domestic poultry (*5*).

Preemptive culling creates a major concern with regard to compensation. In Nigeria, for example, affected farmers have yet to be compensated >50 million Nigerian Naira (>US$ 0.4 million) because of the ministry's cash flow problems (*6*). On the other hand, US poultry farmers who participate in a US Department of Agriculture (USDA) program to prevent the spread of disease would be fully compensated for loss of poultry and equipment if even a low-pathogenic strain of avian influenza was found in the United States (*7*). This rule not only strengthens US protection against avian influenza but also minimizes any negative effect on the US poultry trade.

As discussed by the World Bank (*8*), the situations of these 2 countries raise several questions: Who should pay the compensation? For what should compensation be paid? Who should be compensated? With regard to the first question, each country's government is an exclusive funding source. However, in Nigeria, the amount of compensation overwhelms the government's capacity. Some countries, like Australia, may get additional funding from alternative sources such as private sectors, regional economic groups, or international funds (*9*). Because national resources are often scarce, most developing countries must rely on international donors for a great deal of the funding for compensation programs.

The response to the second question, extent of compensation, varies. In Nigeria, farmers are partially compensated for loss of poultry; however, in the United States, farmers who are part of the USDA program are fully compensated for loss of poultry and equipment. Setting the amount of compensation is difficult and can affect the outcome of culling efforts. In Thailand, to take advantage of the program in which compensation was perceived as high, some farmers reportedly moved infected poultry into previously uninfected areas. In Vietnam, where compensation was perceived as low, culling compliance was poor (*8*).

The last question, who should be compensated, seems straightforward for the United States, where only farmers who participate in the USDA program would be fully compensated. However, H5N1 does not affect only farmers who sign up for such a program. And not all poultry are raised in commercial operations, especially in developing countries. In Thailand, for example, >80% of infected poultry are reportedly raised in backyards (*10*). Reasonable assumptions are that those backyard farmers do not honestly report dying poultry or that they rush sick and dying poultry to market, causing the disease to spread. Additional questions revolve around potential compensation for those who are involved in the poultry industry but who do not own poultry (e.g., poultry processing plant operators and their staff).

Because each country's needs and circumstances differ, building a coherent plan for tackling HPAI is difficult. However, each stakeholder should consider compensation as part of an overall package of prevention, preparedness, and response strategies toward controlling and preventing the spread of HPAI. Because H5N1 does not respect international boundaries, donors worldwide should step forward to support the most affected and vulnerable developing countries.

Sayako Kanamori* and Masamine Jimba*

*University of Tokyo, Tokyo, Japan

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Address for correspondence: Sayako Kanamori, University of Tokyo, Department of International Community Health, 7-3-1 Hongo, Bunkyo-ku Tokyo 113-0033, Japan; email: sayasaya@m.u-tokyo.ac.jp

Frog Virus 3 Infection, Cultured American Bullfrogs

To the Editor: Ranaculture, the practice of farm-raising frogs for scientific and culinary purposes, is practiced in many countries, including the United States (*1*). As with aquaculture, most ranaculture challenges relate to husbandry and disease. In aquaculture, iridovirus infections are reportable and can result in large-scale fish deaths (*2*,*3*). The family *Iridoviridae* is composed of *Iridovirus*, *Chloriridovirus*, *Ranavirus*, and *Lymphocystivirus*. The first 2 infect insects; the latter 2, lower vertebrates (*4*). Infection with frog virus 3 (FV3), the type species of the genus *Ranavirus*, results in edema, hemorrhage, and necrosis of lymphoid tissue, hematopoietic tissue, liver, spleen, and renal tubules (*3*,*5*); mortality rates in free-ranging amphibians are >90% (*6*).

In May 2006, a commercial American bullfrog (*Rana catesbeiana*) ranaculture facility suffered massive (>50%) deaths of frogs that had recently undergone metamorphosis. The facility, with >25 years of experience, uses an on-site breeding colony and an all-in, all-out system, in which cohorts are moved through the system as 1 unit. Well water is used throughout. The breeding colony and larvae are housed in outdoor tanks to expose them to ambient climatic conditions, thought to facilitate breeding and development. Outdoor tanks are covered with mesh to prevent predation by birds. After metamorphosis, animals are moved indoors.

Full necropsies were performed on 3 of the recent metamorphs. A set of fixed tissue sections from all organs was routinely processed for light microscopic examination. An identical set of fresh tissue sections was collected for routine bacterial culture and viral analysis. Bacterial isolates were speciated by using an automated system (Sensititer, Trek Diagnostic Systems, Westlake, OH, USA) or conventional biochemical testing. Virus isolation was performed by using a variety of cell lines; random isolates were verified by electron microscopy. A heminested PCR targeting the major capsid protein gene was performed (*3*), amplicons were sequenced (SeqWright DNA Technology Services, Houston, TX, USA), and a GenBank BLAST search (www.ncbi.nlm.nih.gov/Genbank) was performed.

Pathologic changes in all metamorphs were similar. Gross findings were as follows: irregular gray patches on the skin, cutaneous and enteric erythema, mottled heart and kidneys, pale and friable livers, and enlarged gall bladders. Histologic examination showed lymphoid depletion and necrosis in the thymus and other lymphoid tissues and necrosis in the liver, spleen (Figure), and epidermis.

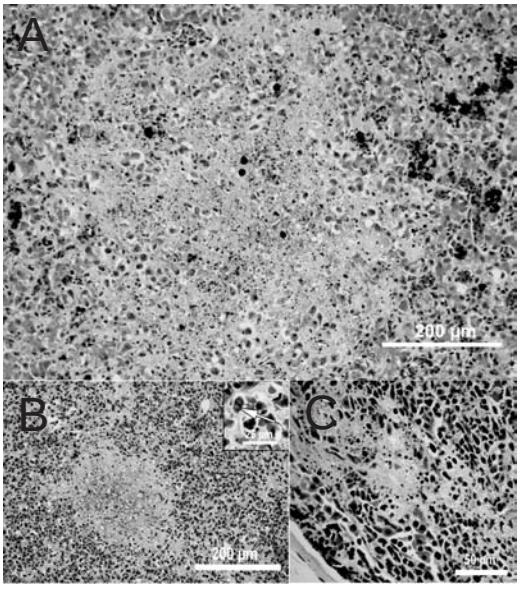

Figure. Light microscopic images of the liver (A), spleen (B), and thymus (C) showing necrosis in an American bullfrog (*Rana catesbeiana*) metamorph infected with frog virus 3. Spleen (B) inset shows intracytoplasmic viral inclusion bodies. Hematoxylin and eosin stain.

Scattered intracytoplasmic inclusion bodies were observed in the spleen (Figure B inset). Epithelial degeneration was noted in the renal tubules. Bacteria were present within the dermal lesions, glomerular tufts and vessels of the kidney, and, rarely, in the spleen and sinusoids of the liver.

Iridovirus was isolated and confirmed by PCR. A BLAST search of the resulting sequence (GenBank accession no. EF101698) showed identity with FV3. *Aeromonas hydrophila* was cultured from the internal organs and dermal lesions. Diagnosis was FV3 infection with a secondary *A. hydrophila* infection. Incidentally, 5 larval bullfrogs obtained from this cohort ≈6 months earlier died of nitrate toxicity 1 month after acquisition. PCR and sequencing identified FV3 (GenBank accession no. EF101697) in these 5 larvae; secondary bacterial invasion was absent.

Susceptibility to FV3 is thought to vary by species and life cycle (*5*–*7*). How the amphibian immune system responds to FV3 infection is not known. Critical periods for infectivity likely include the time before the larval immune system develops, at metamorphosis while the larval immune system is being dismantled, and during periods of exogenous stressors (e.g., movement of the animals from outside to inside tanks). Consequently, we hypothesize that the stress of recent metamorphosis, along with the added stress of movement from outside to inside, likely increased the frogs' susceptibility to FV3.

Further, with lymphoid depletion and multiorgan compromise (necrosis), individual frogs become susceptible to opportunistic pathogens, such as *A. hydrophila*, especially when the innate immune system fails (i.e., skin abrasions). *A. hydrophila* infections alone can result in considerable loss in ranaculture systems (*8*). Unfortunately, specimens often are submitted for bacterial analysis only, not viral testing. Thus, the effects of *Ranavirus* on ranaculture remain unknown. As with free-ranging populations, in which coinfections have been reported (*9*), ranaculture populations that had a diagnosis of *A. hydrophila* may have had an underlying *Ranavirus* infection.

In vertebrates, iridovirus is thought to be transmitted only horizontally (*10*). This ranaculture facility kept frogs separated according to size, to decrease cannibalism. Possible routes of FV3 exposure in this facility are the following: exposure of the larval tank to an infected free-ranging frog, mechanical transmission during routine husbandry, or mechanical transmission by insects. Regardless, at this time the frogs can be treated only for bacterial pathogens; however, early detection and reduction of exogenous stressors may help less-affected bullfrogs clear the virus (*11*) and ultimately reduce loss.

All-in, all-out ranaculture systems may be able to eliminate FV3 infection by preventing exposure of cultured larvae to mechanical vectors and native anurans. Ranaculture systems must eliminate this virus before translocating infected frogs to naive systems. Because amphibians are declining globally, exposure of captive wildlife to free-ranging populations should be minimized.

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Debra L. Miller,*
Sreekumari Rajeev,*
Matthew J. Gray,†
and Charles A. Baldwin*

*University of Georgia, Tifton, Georgia, USA; and †University of Tennessee, Knoxville, Tennessee, USA

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Address for correspondence: Debra L. Miller, University of Georgia, Veterinary Diagnostic and Investigational Laboratory, Tifton, GA 31793, USA: email: millerdl@uga.edu

Pandemic Influenza School Closure Policies

To the Editor: Holmberg et al. (*1*) are rightly concerned that state pandemic plans in the United States represent a patchwork without central coordination or direction. These concerns are particularly relevant for school closure decisions during an influenza pandemic. The US Department of Health and Human Services' checklist regarding school closures gives conflicting messages (*2*). For example, it recommends that schools stay open during a pandemic and develop school-based surveillance systems for absenteeism of students and sick-leave policies for staff and students. It also recommends developing alternate procedures to ensure the continuity of instruction in the event of district-wide school closures. These vague recommendations may reflect the paucity of data to recommend school closure.

To assess the current status of school closure decisions in the United States, I conducted an internet survey of all 50 state health commissioners during the spring of 2006. I asked the respondents 2 questions: "Who makes the school closure decisions in your state?" and "What absenteeism rate, if any, would prompt a school in your state to close during a typical influenza year and/or during a pandemic influenza year?" Of the 44 responding states, I found that school closure decisions were primarily a local-level responsibility in half. Of these 22 states, closure decisions would be made either on a school-by-school or a school district–by–school district basis. Only 6 states indicated that school closure decisions would be made at the state level, and 16 states would have decisions made jointly at the state and local levels (Table).

For a typical influenza season, only 6 states indicated that they close schools if a certain absenteeism rate due to illness were reached. For 5 of these states, the absenteeism rates ranged from 10% to 30%; the sixth state said its schools would close if the rates were anywhere from 7% to 31%. However, only 1 state reported a threshold absenteeism rate for closure during an influenza pandemic. Another state said that it was developing an absenteeism rate that would prompt closure for pandemic influenza. Forty-two states did not have threshold absenteeism rates that would prompt school closures during an influenza pandemic.

In July 2006, the Department for Education and Skills in the United Kingdom published guidelines regarding school closure (D. O'Gorman, pers. comm.). Although the final decision for school closure would lie with local school officials, the national government might advise all schools and childcare facilities to close when a pandemic reached their area to reduce the spread of infection among children (*3*). It is believed that all would comply with closure advice and that use of emergency powers under the UK Civil Contingency Act 2004 to require services to close would not be necessary. If all British schools in an area were advised to close during a pandemic, the situation would be reviewed after a period of time, such as 2 to 3 weeks, by local officials acting on information from the UK government, to decide whether to remain closed.

Although the United States is a nation dedicated to federalism, an uncoordinated approach for community response measures such as school closure decisions could jeopardize our efforts in containing a deadly pandemic. If schools were to remain open until a certain percentage of students and faculty became ill, as they do during typical influenza seasons, then control measures to contain the outbreaks would likely be far more difficult to achieve because a chain of transmission would be established. Some might consider it unethical for schools to stay open in the face of a pandemic with a high death rate. I therefore think a national policy, or at least specific national guidelines, should be developed jointly by the Centers for Disease Control and Prevention and the Department of Education, so that states' school districts can develop rational, coherent, and coordinated closure plans to protect children and communities during an influenza pandemic.

Table. Number of states reporting influenza pandemic school closure policies at various levels, USA*

Region	Local only	State and local	State only
Northeast	4	2	0
South	7	8	2
Midwest	7	3	0
West	4	3	4
Total†	22	16	6

*Northeast: CT, DC, MA, ME, NH, NJ, NY, PA, RI, VT; South: AL, AR, DE, FL, GA, KY, LA, MD, MS, NC, OK, SC, TN, TX, VA, WV; Midwest: IA, IL, IN, KS, MI, MN, MO, ND, NE, OH, SD, WI; West: AK, AZ, CA, CO, HI, ID, MT, NM, NV, OR, UT, WA, WY.
†Six states did not respond.

Laura H. Kahn*

*Princeton University, Princeton, New Jersey, USA

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Available from http://www.teachernet.gov.uk/_doc/9942/FullGuidance.pdf

Address for correspondence: Laura H. Kahn, Program on Science and Global Security, Woodrow Wilson School of Public and International Affairs, Princeton University, 221 Nassau St, 2nd Floor, Princeton, NJ 08542, USA; email: lkahn@princeton.edu

Symptomatic Human Hantavirus in the Americas

To the Editor: In a recent letter (*1*), dos Santos et al. described 3 cases of hantavirus pulmonary syndrome (HPS) from Juquitiba and stated that "the first human cases of symptomatic infection by hantaviruses were reported from Brazil in 1993." However, we described 8 cases of symptomatic hemorrhagic fever with renal syndrome (HFRS) in Recife, Brazil, 5 months before the initial May 1993 report of Sin Nombre virus (SNV)–induced HPS in the United States (*2*). Our report was therefore the first published account of symptomatic hantavirus infections, not just in Brazil but anywhere in the Americas (*3*).

Serum samples from our Brazilian HFRS cases, collected in 1990, were screened by an immunofluorescence assay (IFA) and ELISA for immunoglobulin G, as were the current Brazilian HPS cases (*1*). Two of our patients had an increased immunoglobulin M titer by ELISA (*2*). Rat-transmitted Seoul virus (SEOV) was considered most likely because this was the only hantavirus strain showing clear positive results in IFA (*2*,*3*). All the Recife cases in 1990 had reported likely rat contacts and were initially diagnosed as leptospirosis with acute renal failure and thrombocytopenia, clinical hallmarks of both HFRS and leptospirosis (*3*). We also subsequently found evidence of SEOV infection in 31 (15%) of 201 leptospirosis-suspected acute renal failure cases from Belém, Brazil, confirmed in 1 case with highly specific neutralization tests (*4*). Moreover, as we predicted (*3*), some of the 133 (41%) of 326 urban cases of acute renal failure from Salvador, Brazil, which appeared nonconfirmed for leptospirosis (*5*), were later shown to be caused by SEOV (unpub. data). Finally, of 379 schoolchildren from Salvador at high risk for frequent rat exposure, 13.2% were IFA positive for the Korean prototype Hantaan virus (HTNV) but none for the American SNV (*6*). Because both HTNV and its rodent reservoir are absent from the American biotope, HTNV seroreactivity should be considered a cross-reaction to another related murine antigen; that is to say, the ratborne SEOV.

Wild rats (*Rattus rattus and R. norvegicus*) are the only Old World rodents ubiquitous in the New World and thus a potential source of SEOV infection in the Americas (*3*,*7*). Moreover, the first hantavirus characterized in South America was SEOV, isolated as long ago as 1984 from a rat caught in Belém (7). Furthermore, the first 3 clinical cases of hantavirus infection in the United States were SEOV-induced (Baltimore rat virus) HFRS cases and not HPS (*8*).

The clinical syndromes of HFRS and HPS can appear identical, with pulmonary edema, shock, and renal insufficiency with marked proteinuria and thrombocytopenia (*9*). Moreover, worldwide ELISA testing with a single antigen such as SNV or Puumala virus (PUUV) can result in misleading cross-reactions, since both viruses are genetically related. Although this SNV-PUUV cross-reactivity enabled the first recognition of HPS cases in the New World 14 years ago, this may now lead to the wrong clinical diagnosis and reinforces the need for specific tests such as neutralization tests or reverse transcription–PCR. Although not as lethal and probably not so frequent as HPS, SEOV-induced HFRS may still be greatly underestimated in the Americas, or misdiagnosed as leptospirosis.

Jan Clement,* Guy H Neild,† Piet Maes,* Herwig Leirs,‡ Patrick Matthys,* and Marc Van Ranst*

*University of Leuven, Leuven, Belgium; †Royal Free Hospital, London, United Kingdom; and ‡University of Antwerp, Antwerp, Belgium

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Address for correspondence: Jan Clement, Hantavirus Reference Centre, Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B3000 Leuven, Belgium; email: jan.clement@uz.kuleuven.ac.be

Echinococcosis Risk among Domestic Definitive Hosts, Japan

To the Editor: Echinococcosis is a serious parasitic zoonosis in the Northern Hemisphere. In Japan, it is characterized by alveolar, hepatic, and cerebral disorders in humans caused by the larval form (metacestode) of the tapeworm *Echinococcus multilocularis*. The life cycle of the parasite is maintained in the wild by gray-backed voles, *Clethrionomys rufocanus*, as intermediate hosts, and by red foxes, *Vulpes vulpes*, as definitive hosts. Humans are infected by ingestion of the parasite eggs, mainly through water contaminated with the feces of wild red foxes, which have an estimated infection prevalence of 54%–56% (*1*).

The echinococcosis-endemic area in Japan is restricted to the northern island of Hokkaido, although sporadic human cases have been reported on other islands (*2*), and infected pigs have been documented on the main island of Honshu (*3*). While the threat of echinococcosis spreading into Honshu had raised fears, an emergent concern is the possible role of domestic dogs in dispersing the disease from disease-endemic areas during relocation of residences by owners or when accompanying owners during domestic travel.

In September 2005, a stray dog in Saitama prefecture in mainland Honshu was found to be positive for *E. multilocularis* infection by PCR (mitochondria 12S RNA gene) (Y. Morishima, pers. comm.). The sequence was identical to the Hokkaido isolate (GenBank accession no. AB244598). This raised an alarm because the area in which the infection was found is adjacent to the Tokyo metropolis, the most populous zone in Japan. Reports also claimed that 2 of 69 dogs moved from Hokkaido to Honshu were positive for *E. multilocularis* by coproantigen examination (*4*).

Nearly 10,000 pet dogs were estimated to have been transported in 1 year to and from Honshu and Hokkaido by planes and ferries; this presumably included up to 30 *E. multilocularis*–infected pet dogs per year (*5*). Even so, no compulsory quarantine or *Echinococcus* examination is enforced for dogs transported within Japan. A compulsory requirement of a certificate from a veterinarian stating that the animal has been treated with praziquantel 3–4 days before traveling would be a helpful preventive measure. As part of an amendment to the Infectious Disease Law in Japan, *E. multilocularis* infection was included among the 4th Category Diseases (*6*). Thus, since October 2004, it has been mandatory for veterinarians who have diagnosed echinococcosis in dogs to report each case to health authorities, the first national reporting system of its kind worldwide.

Our laboratory established the Forum on Environment and Animals (FEA) to meet the demand for accurate and rapid diagnosis of echinococcosis in domestic dogs. FEA is a hub for veterinary practitioners around the country for confirmation of *E. multilocularis* infection in definitive hosts, especially dogs but also cats. Feces submitted are from dogs and cats that are suspected to be infected and that wander or walk in parks and woodlands and likely prey on wild rodents. Examinations are performed weekly, and results are immediately forwarded to the submitting veterinarians. Before examination, fecal samples are sterilized by heating for 12 hours at 70°C. Fecal egg examination is conducted by using centrifugal flotation (*7*) with sucrose solution with a specific gravity of 1.27. Sandwich ELISA using a monoclonal antibody EmA9 (*8*) is used for *E. multilocularis* coproantigen detection. Egg- and ELISA-positive fecal samples from dogs are subjected to PCR amplification (mitochondria 12S RNA gene) (*9*).

The Table presents data of samples from both dogs and cats examined by FEA from April 2004 through August 2005. A total of 1,460 domestic dogs were examined, and 4 (0.27%) were confirmed positive to echinococcosis by PCR, all from Hokkaido. Test results from eggs detected in cat feces suggested these animals were infected with *Taenia taeniaeformis*, a cat tapeworm, rather than *E. multilocularis*, because coproantigen ELISA results were negative and an ELISA-positive sample did not contain eggs.

To our knowledge, this survey registered the greatest number of domestic dogs examined recently in Japan

Table. Prevalence of echinococcosis in definitive hosts subjected to fecal egg examination, ELISA coproantigen test, and PCR copro-DNA detection, Japan

Species	No. samples	Positive samples (%)		
		Egg examination	ELISA	PCR
Dogs	1,460	3 (0.20)	6 (0.41)	4 (0.27)
Cats	128	4 (3.12)	1 (0.78)	ND*
Total	1,588	7 (0.44)	7 (0.44)	–

*ND, not done.

for echinococcosis. Confirmed cases of infection in dogs further showed the potential threat of domestic dogs transmitting *E. multilocularis* to humans in this region, as well as the potential for dispersal to other islands of Japan if proper preventive measures are not implemented.

A previous report of necropsy examinations of 9,849 dogs from 1966 to 1999 showed a prevalence of 1.0% (*10*). Although necropsy is considered the most reliable method to diagnose *E. multilocularis* in definitive hosts, it is not applicable for live animals such as domestic dogs and cats. Fecal egg examination is generally used; however, infection is difficult to confirm because the morphology of taeniid eggs is indistinguishable from those of *E. multilocularis*, and eggs are excreted intermittently even after the worms mature. Coproantigen detection had proven useful for primary screening and was documented to have 94.9% sensitivity and 100% specificity for echinococcosis in wild red foxes in Hokkaido (*1*). The combined egg examination, ELISA, and PCR methods we used showed an accurate and rapid diagnosis in domestic dogs, which is important for immediate reporting, treatment, and action to safeguard dog owners.

Masao Kamiya,*
Jose Trinipil Lagapa,*†‡
Sumiya Ganzorig,§
Fumio Kobayashi,§
Nariaki Nonaka,‡
and Yuzaburo Oku‡

*Rakuno Gakuen University, Hokkaido, Japan; †Central Mindanao University, Musuan, the Philippines; ‡Hokkaido University, Hokkaido, Japan; and §Forum on Environment and Animals, Hokkaido, Japan

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Address for correspondence: Masao Kamiya, OIE Reference Laboratory for Echinococcosis and Laboratory of Environmental Zoology, Department of Biosphere and Environmental Sciences, Faculty of Environmental Systems, Rakuno Gakuen University, Bunkyodai-midorimachi 582, Ebetsu, Hokkaido 069-8501, Japan; email: mkamiya@rakuno.ac.jp

Maculopathy and Dengue

To the Editors: We thank Su and Chee (*1*) for their interest in our article, "Retinal Hemorrhages in 4 Patients with Dengue Fever" (*2*). We reported the findings of this small case series to highlight the presence of retinal hemorrhage as a manifestation of ophthalmic complication in patients with dengue fever. We wanted to describe characteristic clinical features (such as association of onset of visual symptoms with resolution of fever and nadir of thrombocytopenia) and propose epidemiologic explanations for the sudden rise in the incidence of observed ocular complications of dengue fever in our population. Our article did not attempt to conclude that the retinal hemorrhages were responsible for the patients' visual symptoms, as suggested by Su et al. In fact, we stated that in all 4 patients "fundoscopic examination showed macular hemorrhages and exudative maculopathy."

The range of dengue-related ophthalmic complication is still being investigated, and we agree with Su and Chee that other ophthalmic manifestations may occur in patients with dengue fever. In a retrospective observational case series involving 22 eyes of 13 patients with visual impairment from dengue infection, carried out in our hospital, Chan et al. (*3*) found evidence of retinal hemorrhage, macular edema, cotton wool spots, retinal vasculitis, exudative retinal detachment, and anterior uveitis. Therefore, physicians and ophthalmologists should be aware of the possibilities of ophthalmic complications in the management of patients with dengue fever.

Augustinus Laude,*
Maciej Piotr Chlebicki,*
Brenda Ang,*
and Timothy Barkham*

*Tan Tock Seng Hospital, Singapore

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Address for correspondence: Augustinus Laude, Tan Tock Seng Hospital, 11, Jalan Tan Tock Seng, Singapore 308433, Singapore; email: laude_augustinus@ttsh.com.sg

Enterohemorrhagic *Escherichia coli* Excretion by Child and Her Cat

To the Editor: Enterohemorrhagic *Escherichia coli* (EHEC) can cause severe hemorrhagic colitis characterized by gastrointestinal symptoms and bloody diarrhea as well as hemolytic uremic syndrome (*1*). Cattle and small ruminants are the major natural reservoir of these foodborne pathogens (*1*,*2*). Human infections may also develop after direct contact with cows, goats, sheep, and deer (*1*). Although domestic dogs and cats are known as rare EHEC carriers (*3*,*4*), no human EHEC infections associated with pet contact have been reported. Here we report the first case of an EHEC strain infecting both a child and her domestic cat.

A 2-year-old girl with bloody diarrhea and vomiting subsequently tested positive for EHEC serotype O145:H–. The isolated strain harbored the pathogenicity-associated genes *stx1*, *stx2*, *eae*, and *hly*, as tested by PCR. An enterohemolytic phenotype was also present. After notification of the local health authority, a rigorous search for the possible source of the girl's infection was started. When asked for instances of animal contact, her parents mentioned the family cat, which the girl often handled. The cat is restricted to the house, has no contact with other animals, and is fed only canned catfood. The animal strictly uses a litter box, which is cleaned regularly by the parents. No gastrointestinal symptoms in the cat were recorded. Repeated stool samples from the cat grew a strain of EHEC O145:H– that showed the identical pathogenicity gene pattern as the girl's isolate. Moreover, a restriction fragment length polymorphism analysis proved the clonal identity of both strains. Because both the girl and the cat continuously excreted the EHEC strain, the cat was assumed to be a possible source of the girl's infection or reinfection. The cat's infection was treated with probiotics, but the child's EHEC positivity did not change. After 3 months, the girl spontaneously stopped excreting EHEC, while the cat's stool samples remained EHEC positive. The cat was then treated by peroral autovaccination with the heat-inactivated EHEC strain for 10 consecutive days and subsequently stopped shedding EHEC. In the Table, the clinical course and laboratory findings of both girl and cat are summarized.

To our knowledge, this case is the first documented of an EHEC strain's affecting both a human and a domestic cat. Both excreted EHEC for ≈3 months. Although the girl had vomiting and diarrhea, the cat was asymptomatic. Several possibilities regarding the infectious process can be noted. First, the girl might have contracted the disease from her asymptomatic pet. Although in a study on *eae*-positive *E. coli* strains, ≈6% of the investigated 62 cats tested positive, none of these cats was infected with EHEC serotype O145:H– (*3*); this finding indicates that in our case the cat might not have been the direct source for the girl's infection. Moreover, foodborne transmission to the cat seems unlikely because it was exclusively fed with canned food that was heated during preparation. Second, the cat might have been infected by the girl. Although the prevalence of EHEC serotype O145:H– is relatively low, it ranks among the 6 most often isolated non–O157 EHEC strains in human infections, accounting for 5%–7% of all non–O157 EHEC strains in prevalence studies in Finland (*5*), Germany (*6*), and the United States (*2*,*7*). A similar epidemiologic pattern for EHEC serotype O145:H– is seen in

Table. Clinical picture and isolation of EHEC serotype O145:H– from stool samples of child and her cat*

Date	Girl	Cat
Dec 1, 2004	Vomiting and diarrhea	
Dec 9	Tested positive	ND
Dec 22	Tested positive	Tested positive
Dec 28	Tested positive	ND
Jan 10, 2005	Tested positive	ND
Jan 17	Tested positive	ND
Jan 21	Tested negative	ND
Jan 24	Tested positive	Tested positive, treated with probiotics
Feb 1	Tested positive	ND
Mar 4	Tested negative	ND
Mar 12	Tested negative	ND
Apr 25	ND	Tested positive
Jun 25–Jul 4	ND	Autovaccination
Jul 29	ND	Tested negative
Aug 11	ND	Tested negative

*EHEC, enterohemorrhagic *Escherichia coli*; positive and negative refer to the isolation of EHEC serotype O145:H– ; ND, no testing was done.

cattle (*2*,*8*). Taken together, the prevalence of EHEC serotype O145:H– in cats, humans, and cattle might indicate that the girl was probably more likely the infection source for the cat than vice versa. Third, a cycle of mutual infection and reinfection between the girl and her pet cat cannot be ruled out. Although the excretion rate for EHEC changes over time and EHEC can therefore remain undetected in stool samples while still present within the patient, the child tested EHEC negative for a short period. Despite all the precautions taken, the girl may have been reinfected by the cat.

This case illustrates several issues: 1) domestic animals such as cats (*3*), dogs (*3*,*4*), and rabbits (*9*) may serve as reservoirs for EHEC, irrespective of whether they are the primary or secondary source for these bacteria; 2) domestic cats as carriers may excrete EHEC for a prolonged period; 3) autovaccination may be effective for treating EHEC-infected animals; and 4) fondness for pets may be problematic: although EHEC O145:H– is among the 4 most often isolated EHEC serotypes associated with severe colitis or life-threatening hemolytic uremic syndrome (*10*), the girl's parents, after weighing the infectious risks against the psychological benefits for both their daughter and her feline companion, decided not to send the cat to an animal shelter until its EHEC infection disappeared.

Ulrich Busch,*
Stefan Hörmansdorfer,*
Stephan Schranner,† Ingrid Huber,*
Karl-Heinz Bogner,*
and Andreas Sing*

*Bavarian Health and Food Safety Authority, Oberschleißheim, Germany; and †Veterinary Inspection Office, Landshut, Germany

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Address for correspondence: Andreas Sing, MA DTM&H, Bavarian Health and Food Safety Authority, Veterinärstraße 2, 85764 Oberschleißheim, Germany; email: andreas.sing@lgl.bayern.de

Misdiagnosing Melioidosis

To the Editor: Melioidosis is endemic in southern and Southeast Asia and northern Australia. Although relatively few indigenous cases are recognized in the Indian subcontinent, a substantial proportion of cases imported into the United Kingdom originate there, probably reflecting patterns of immigration and travel, and underdiagnosis within the Indian subcontinent (*1*–*3*).

A 33-year-old woman spent 3 months in India. Shortly after arriving there, fever, myalgia, rigors, pharyngitis, and tender cervical lymphadenopathy developed. After she received antimicrobial agents, her symptoms initially improved, but in September 2005, 1 week after returning to the United Kingdom, she visited her general practitioner with recurrent fever and increasingly painful cervical lymphadenopathy. She was given a course of oral co-amoxiclav 625 mg 3× daily. However, the following week she visited the emergency department of her local hospital with left-sided suppurative cervical lymphadenitis. Pus aspirated from the lymph node grew an aminoglycoside-resistant "pseudomonad" identified as *Pseudomonas fluorescens* (API20NE profile 1056554), assumed to be a contaminant. She was discharged home to complete a further 10-day course of co-amoxiclav.

One month later, the patient again went to the emergency department, this time with a submental abscess. An otolaryngology consultation was sought, and the abscess was incised and drained. Although tuberculosis was suspected, no acid-fast bacilli were identified, and cultures were negative for mycobacteria; histologic examination showed noncaseating granulomata. Culture of fluid from the submental collection again yielded an aminoglycoside-resistant pseudomonad, however. At this point misidentifi-

cation was suspected, and the isolate, which had a characteristic colony form on Ashdown's Medium, microscopic appearance (Figure, panel A), API20NE profile (1556574), and fatty acid profile, was identified as *Burkholderia pseudomallei*, the etiologic agent of melioidosis.

The patient had no relevant past medical history. Before immigrating to the United Kingdom 3 years earlier, she had lived in Tanjore, a rice-farming area of Tamil Nadu. She had stayed with family there during her recent trip, which coincided with the monsoon season, but she denied rural travel, fresh water contact, or skin abrasions. On examination, she was obese with acanthosis nigricans and tender cervical lymphadenopathy. Blood tests showed a mild microcytosis, low ferritin level, and erythrocyte sedimentation rate 40 mm/h; serum biochemistry and levels of C-reactive protein, fasting glucose, and hemoglobin by electrophoresis were normal. Two blood cultures were negative. Results of chest and abdominal imaging were normal. The patient was treated with intravenous ceftazidime for 10 days and oral co-trimoxazole for 4 months. She remains well.

B. pseudomallei serologic tests, performed subsequently, showed negative results by ELISA against the standard laboratory strain (204). However, when the assay was repeated using the patient's own isolate, the result was positive (immunoglobulin G titer 4,000). Comparison of lipopolysaccharide (LPS) antigens from the 2 strains by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting showed that they differed in O-repeating units (Figure, panel B).

B. pseudomallei is an aerobic, gram-negative, environmental saprophyte ubiquitous in soil and surface water (e.g., paddy fields) in disease-endemic areas. Acquisition may occur through skin abrasions, aspiration of fresh water, inhalation, and possibly ingestion and may occasionally occur in the laboratory. An association between severe respiratory melioidosis and heavy monsoonal rains suggests that inhalation has previously been underrecognized as a route of infection (*4*); this is the likely mode in this case.

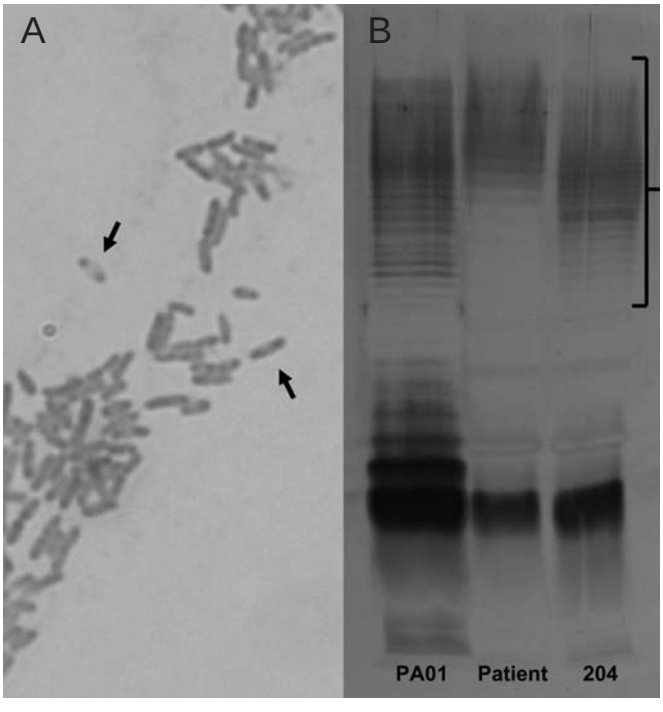

Figure. A) Gram stain of pus from the patient's submental collection, showing the characteristic safety-pin pattern (arrows) of bipolar staining. B) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of lipopolysaccharide (LPS) antigens from the patient and *Burkholderia pseudomallei* reference strain (204), showing different O-repeating units (bracket). A control isolate of *Pseudomonas aeruginosa* LPS (PA01) is shown for comparison.

Many infections are initially subclinical but may result in latency and delayed manifestations, even after several decades. Clinical signs and symptoms include septicemia, cavitating pneumonia, bone and soft tissue infections, disseminated abscesses, mycotic aneurysms, lymphadenitis, and childhood parotitis. Most patients have an underlying predisposition to infection, especially diabetes, renal disease, alcoholism, and thalassemia, but in the largest Indian case series 50% patients had no traditional risk factors, as with our patient (*5*). *B. pseudomallei* is a category B potential bioterrorism agent.

Limited awareness of the disease, confusion with other conditions such as tuberculosis, and laboratory constraints all probably contribute to underdiagnosis of melioidosis in many areas (*6*). However, accurate diagnosis is important because septicemic melioidosis may be rapidly fatal, *B. pseudomallei* is intrinsically resistant to many antimicrobial agents, and prolonged treatment is usually required to minimize relapse. Diagnosis is usually by culture from sterile sites. Laboratory misidentification is not uncommon and occurred in this case because the diagnosis was not considered. Isolation of aminoglycoside-resistant pseudomonads in patients from disease-endemic areas should always prompt consideration of melioidosis and accurate identification. PCR is an emerging diagnostic tool not yet extensively validated (*7*).

The role of serology in diagnosis is limited by high background seropositivity rates in disease-endemic areas. No standardized serologic test is inter-

nationally agreed upon. This case illustrates another potential pitfall in melioidosis serodiagnosis. Most isolates express a conserved LPS antigen, which allows use of a single reference strain for determination of anti-LPS antibodies (*8*). However, because some strains express different LPS antigens, serologic tests must be performed with the patient's own strain.

This case illustrates potential pitfalls in diagnosing melioidosis, which requires clinical and laboratory awareness and knowledge of its geographic distribution. LPS-based serologic assays should use a range of isolates representative of known LPS types.

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A. J. Brent and R. Handy had clinical responsibility for the patient. R. Handy and P.C. Matthews made the initial microbiologic diagnosis of melioidosis, and T.L. Pitt confirmed the isolate as *Burkholderia pseudomallei*. T.L. Pitt performed the serology and SDS-PAGE analysis of lipopolysaccharide antigens. All authors contributed to preparation of the manuscript. A.J. Brent is guarantor for the article, had full access to all the clinical and microbiologic data, and had final responsibility for the decision to submit for publication.

Andrew J. Brent,* Philippa C. Matthews,* David A. Dance,† Tyrone L. Pitt,‡ and Rupert Handy§

*John Radcliffe Hospital, Headley Way, Headington, Oxford, United Kingdom; †Health Protection Agency (South West), Plymouth, Devon, United Kingdom; ‡Laboratory of HealthCare Associated Infection, London, United Kingdom; and §Heatherwood and Wexham Park Hospitals, Wexham, Slough, United Kingdom

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Address for correspondence: Andrew J. Brent, Nuffield Department of Infectious Diseases & Microbiology, John Radcliffe Hospital, Headington, Oxford, United Kingdom OX3 9DU; email: dr.a.brent@ gmail.com

Subclinical *Plasmodium falciparum* Infection and HIV-1 Viral Load

To the Editor: Studies indicate that *Plasmodium falciparum* infection increases HIV replication in adults (*1*,*2*). Although malaria-related illness and death are more common in children, and HIV-1 generally progresses faster in children than in adults (*3*,*4*), to our knowledge the effect of intermittent malaria on HIV-1 viral load has not been directly explored in children. To investigate this issue, we monitored HIV-positive infants from a 1996–2001 birth cohort study in Kisumu, Kenya, a *P. falciparum*–holoendemic area.

Study design and methods have been described elsewhere (*5*,*6*). Twenty-four children that were perinatally infected with HIV were included in this substudy. During monthly visits during the child's first 2 years of life, malaria and HIV incidence were recorded (*5*,*6*). Both children with malaria-positive blood smears and those with fever but no smear result available were treated with sulfadoxine-pyrimethamine according to national guidelines. At the time of this substudy, none of the study participants were taking antiretroviral drugs.

HIV and malaria diagnoses were determined by using standard methods (*5*–*7*). To reduce the chance of including infants infected through breast-feeding, perinatal infection was defined as ≥2 consecutive HIV-positive tests, with the first positive PCR result by 4 months of age (*7*). The so-called baseline viral load was the pre-malaria value measured 1 month before the first observation in the analysis. To be included in the analysis, follow-up visits had to have data available on the current and previous months' viral load and malaria status and occur at roughly monthly intervals at ≥4 months of age.

Malaria parasites were found at 53 of 146 visits in the month before viral load measurement, although at 89% of visits in which children were malaria-positive, the children's samples had <1,000 parasites/μL, and in only 13% of visits in which children had parasitemia did they also have fever (*8*). Median number of observations per child was 7 (range 2–18). No significant demographic or clinical differences were found between HIV-positive children in this substudy and those enrolled in the full cohort (data not shown).

Clinical and demographic variables were evaluated in univariate repeated measures analysis to determine associations with log-transformed HIV-1 viral load. Age and

baseline viral load were strong predictors of current load (Table). Although not statistically significant, clearing the previous month's malaria infection was associated with a drop in viral load (Table, p = 0.09). It was not possible to distinguish between the effects of treatment versus malaria clearance because 87% of malaria infections were treated with antimalarial drugs. However, viral load increased in those incorrectly treated for malaria presumptively (Table).

After adjusting for age and baseline viral load, we assessed $\log_{10}$ HIV viral load in relation to malaria clearance, persistence, absence, or new infection using a repeated measures model with autoregressive covariance structure. No differences were statistically significant, although clearing an infection versus no malaria had a 0.22 log viral load decrease (Table, p = 0.10). When 15 malaria episodes with malaria-free visits 1 month before and after the episode were compared, mean difference (signed-rank test) in viral load "before" and "after" malaria was not significant.

Our findings suggest that low-density malaria infection may not dramatically affect plasma HIV-1 levels in infants. This finding is similar to results of studies of perinatally HIV-infected children in which, although viral loads were unavailable, number of malaria episodes did not significantly affect development of AIDS-related symptoms (*9*,*10*). While clinical malaria leads to at least short-term HIV viral load increases in adults (*1*,*2*), the effect of subclinical malaria is unclear, and even less is known about coinfection in children. Children usually have higher baseline viral loads than adults; thus, the relative effect of malaria on viral load may not be as great. To reduce the impact of passively transferred maternal antibodies, analyses were done on visits after the child was 4 months old. However, lack of fully acquired antimalarial immunity may have led to different HIV/malaria interactions than seen in adults.

Viral load increased in infants that were incorrectly treated presumptively (due to fever) for malaria (Table). Most of these children were found to have other infections. Fever in malaria-endemic areas is often assumed to be malaria-related, but delay in treatment of nonmalarial infections may be harmful in HIV-infected children

Our assessment was limited in size and duration. Furthermore, in attempting to provide optimal patient care through conducting monthly surveillance and encouraging mothers to bring children in during febrile episodes, ability to assess the effect of high-density malaria was diminished because parasitemia levels never reached clinically significant levels. Finally, because malaria was diagnosed by microscopy, rather than PCR, some subclinical malaria infections may have been misclassified as malaria-negative.

Although we found no evidence of an association between subclinical,

Table. Associations with log HIV-1 viral load in infants*

Factor	No.†	Predicted beta (SE)	p value
Baseline viral load			
Log viral load per mL	146	**0.65 (0.09)**	**<0.01**
Documented fever			
Temperature ≥37°C	23	–0.02 (0.11)	0.85
Previous visit documented fever			
Temperature ≥37°C	18	0.07 (0.14)	0.62
Vaccination within 2 weeks of visit			
Yes	15	0.14 (0.14)	0.34
Vaccination within 2 weeks of previous visit			
Yes	18	0.09 (0.12)	0.46
Pneumonia			
Present	22	0.01 (0.13)	0.93
Anemia			
Hemoglobin <8 g/dL	25	–0.25 (0.16)	0.15
Anemia during previous visit			
Hemoglobin <8 g/dL	27	0.01 (0.13)	0.95
Antimalarial drugs received at or since previous visit			
Antimalarial drugs given	59	0.02 (0.10)	0.84
Malaria status current visit			
Parasitemia positive	39	–0.07 (0.10)	0.05
Malaria status previous visit			
Parasitemia positive	53	–0.14 (0.09)	0.14
Malaria status and antimalarial drug use received at or since previous visit			**<0.01**
Treated parasitemia	46	–0.11 (0.09)	0.25
Did not treat parasitemia	7	–0.002 (0.17)	0.99
Treated, no parasitemia	13	**0.28 (0.10)**	**<0.01**
Did not treat, no parasitemia	80	Reference	
Malaria dynamics (previous vs. current visit)			0.36
Cleared infection‡ (+ –)	29	–0.20 (0.11)	0.09
Reinfection/persistence (+ +)	24	–0.16 (0.15)	0.28
New infection (– +)	15	–0.18 (0.14)	0.20
No malaria‡ (– –)	78	Reference	
Age, mo			
4–9	73	**–0.39 (0.13)**	**<0.01**
10–24	73	Reference	

*Statistically significant associations (p<0.05) are indicated in **boldface**.
†Total sample size was 146 follow-up visits. Number of visits with the indicated characteristic are listed. Sample size was 145 for pneumonia and anemia and 144 for anemia during previous visit.
‡After multiple comparisons (Tukey-Kramer) between groups in a model of the effect of malaria on log HIV viral load adjusted for age and baseline viral load, no statistically significant differences were found, although clearing an infection versus no malaria had a p value of 0.10.

low-density malaria and infant HIV-1 viral load, the consequences of high-density or clinical malaria need to be explored. If clinical malaria in infants increases HIV-1 viral load as it does in adults (*1*,*2*), our study underscores dual benefits of malaria treatment in the context of HIV: 1) keeping malaria in check, and 2) preventing an increase in HIV viral load. Ethical issues prevent prospective studies to assess the impact of coinfection early in life, but alternatives include using animal models or stored specimens.

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Kimberly C. Brouwer,*
Lisa B. Mirel,* Chunfu Yang,*
Renu B. Lal,*
Margarette S. Kolczak,*
Anne M. Van Eijk,† John Ayisi,†
Juliana A. Otieno,‡
Bernard L. Nahlen,*§
Richard Steketee,* Ya Ping Shi,*†
and Altaf A. Lal*

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Kenya Medical Research Institute, Kisumu, Kenya; ‡New Nyanza Provincial General Hospital, Kisumu, Kenya; and §World Health Organization, Geneva, Switzerland

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Address for correspondence: Kimberly C. Brouwer, Division of International Health and Cross-Cultural Medicine, Department of Family and Preventive Medicine, School of Medicine, University of California, San Diego, 9500 Gilman Dr, MC 0622, La Jolla, CA 93093-0622, USA; email: kbrouwer@ucsd.edu

African Tickbite Fever in Travelers, Swaziland

To the Editor: African tickbite fever (ATBF), which is caused by *Rickettsia africae*, is well documented in travelers to southern Africa (*1*–*3*) and transmitted by ungulate ticks of the genus *Amblyomma*. Positive serologic results were reported in 9% of patients (*1*) and 11% of travelers (*4*) from southern Africa. We report an outbreak of ATBF with an attack rate of 100% among 12 Dutch travelers to Swaziland.

The 12 travelers (9 male and 3 female) visited Mkhaya Game Reserve in Swaziland in May 2003 for several days. Upon retuning to the Netherlands, they consulted our clinic for assessment for fever, malaise, and skin eruptions. Epidemiologic and clinical data were obtained after the patients provided informed consent. All symptomatic patients were treated before serum samples were collected.

Acute-phase and convalescence-phase serum samples were obtained from 8 patients at 3 and 9 weeks, respectively, after symptoms were reported. Only convalescent-phase serum samples were obtained from the other 4 patients. Serologic assays were conducted for screening and confirmation in Rotterdam, the Netherlands (Department of Virology, Erasmus University Hospital) and Marseille, France (Unité des Rickettsies, Faculté de Médecine, Université de la Mediterranée), respectively.

In Rotterdam, immunofluorescence assays for immunoglobulin G (IgG) and IgM against *R. conorii*, *R. typhi*, and *R. rickettsii* were performed with multiwell slides on which antigens were fixed (Panbio Inc., Columbia, MD, USA). Serum samples with fluorescent rickettsiae at dilutions ≥1:32 were considered positive.

In Marseille, a microimmunofluorescence assay for IgG and IgM against *R. africae*, other members of the spotted fever group, and *R. typhi* of the typhus biogroup was used. Western blotting for *R. africae* and *R. conorii* was performed with reactive serum samples and repeated after cross-adsorption that removed only antibodies to *R. conorii* (*5*). Serologic evidence for infection with *R. africae* was defined as 1) seroconversion; 2) IgG titers >64, IgM titers >32, or both, with IgG and IgM titers >2 dilutions higher than any of the other tested spotted fever group rickettsial antigens; 3) a Western blot profile that showed *R. africae*–specific antibodies; and 4) cross-adsorption assays that showed homologous antibodies against *R. africae* (*1*).

All 12 travelers had a diagnosis of ATBF. Epidemiologic, clinical, and serologic results are shown in the Table. Two patients had a history of a tickbite. Lymphadenopathy in the groin was the only clinical sign observed in 2 other patients. For all 10 patients with symptoms, the symptoms abated within a few days after treatment with doxycycline, 100 mg orally twice a day (5 patients) for 7 days, or ciprofloxacin, 500 mg orally twice a day (5 patients) for 7 days. No relapses or complications were noted 1 year later.

Assays in both locations showed serologic reactivity against *R. conorii* and *R. rickettsiae*. Specific antibodies against *R. africae* were detected by Western blot in 8 patients (Table). All 12 travelers were infected with *R. africae*. In 3 other patients, immunofluorescence assays demonstrated seroconversion for specific antibodies. One patient with no clinical symptoms had low IgG (32) and IgM (16) titers against rickettsiae by immunofluorescence and IgG by Western blot.

Tick vectors of *R. africae* attack humans throughout the year. The proportion of patients having multiple eschars, which indicate the aggressive behavior of the tick, varies from 21% (*6*) to 54% (*2*). The 100% attack rate observed in this study emphasizes the risk for ATBF in sub-Saharan travelers. In our study group, only 2 persons had multiple eschars, but serologic analysis showed that all patients were infected with *R. africae*. Most cases of ATBF have a benign and self-limiting course with fever, headache, myalgia, and a skin rash. However, patients who are not treated show prolonged fever, reactive arthritis, and subacute neuropathy (*7*).

The long-term sequelae of ATBF remain to be established. Early treatment would not likely have prevented these complications. Jensenius et al. reported that travel from November through April was a risk factor for ATBF (*1*). The travelers in our study visited Swaziland in May. We speculate that tick bites were likely caused by larvae or nymphs, which are often

Table. Clinical and serologic characteristics of 12 travelers with African tickbite fever, Swaziland, 2003*

					Rotterdam, the Netherlands		Marseille, France			
T	Sex/ age, y	Fever/ headache/ myalgia/ rash	Tickbite/ eschar site/ lymph. site	Sera	*Rickettsia conorii* IgG/IgM†	*R. rickettsii* IgG/IgM†	*R. conorii* IgG/IgM†	*R. africae* IgG/IgM†	WB	WB ads.
1	F/47	Y/N/Y/Y	N/N/groin	A	0/32	32/32	0/0	0/0	NT	NT
				C	>128/>128	>128/32	128/0	128/0	+	Ra
2	M/14	Y/Y/N/N	Y/foot/groin	A	>64/>64	>64/>64	32/0	64/0	+	Ra
				C	>128/32	>128/16	256/0	256/0	NT	NT
3	M/13	N/N/N/N	N/N/N	A	0/0	32/0	0/16	0/16	+	NC
				C	0/0	0/0	0/16	0/16	NT	NT
4	M/10	N/N/N/N	N/N/groin	A	>64/>64	>64/>64	0/16	0/32	+	Ra
				C	>128/32	>128/16	0/64	0/64	NT	NT
5	M/50	Y/Y/N/Y	N/N/groin	A	0/0	0/0	0/0	0/0	NT	NT
				C	>128/>128	>128/32	64/8	64/8	+	NC
6	M/13	N/N/N/N	N/N/groin	A	0/0	0/0	0/0	0/0	NT	NT
				C	>128/16	>128/16	32/16	32/16	+	Ra
7	M/11	Y/N/N/N	N/N/retro.	A	0/0	32/0	0/0	0/0	NT	NT
				C	0/>128	16/32	0/32	0/32	+	NC
8	F/47	Y/Y/Y/Y	N/mult./groin	A	0/0	32/0	0/16	0/16	+	NC
				C	>128/16	>128/0	32/8	32/8	NT	NT
9	M/5	Y/N/N/N	Y/thumb/ axillary	C	NT/NT	NT/NT	0/0	0/0	Ra	NT
10	M/44	Y/Y/Y/N	N/shoulder/ axillary	C	32/32	>128/>128	0/0	0/0	+	Ra
11	F/10	N/N/N/N	N/N/trunk	C	0/0	0/0	0/0	0/0	+	Ra
12	M/50	N/N/N/N	N/N/N	C	0/0	0/0	0/8	0/16	+	Ra

*T, traveler; lymph., lymphadenopathy; IgG, immunoglobulin G; IgM, immunoglobulin M; WB = Western blot; WB ads., WB after cross-adsorption that removed antibodies to *R. conorii*; Y, yes; N, no; A, acute phase; NT, not tested; C, convalescent phase; +, positive for *R. africae* and *R. conorii*; Ra, positive for *R. africae*; NC, not conclusive; retro., retroauricular; mult., multiple.
†Ratio of titers.

unrecognized stages. Many affected travelers may not seek medical attention or may have received a wrong diagnosis. Therefore, surveillance based only on reported cases is likely to underestimate the true incidence of travel-associated *R. africae* infection.

Paul M. Oostvogel,*
Gerard J. van Doornum,†
Russouw Ferreira,‡
Jacqueline Vink,*
Florence Fenollar,§
and Didier Raoult§

*Medical Center Haaglanden, The Hague, the Netherlands; †Erasmus University Hospital, Rotterdam, the Netherlands; ‡Skukuza, Republic of South Africa; and §Université de la Mediterranée, Marseille, France

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Address for correspondence: Paul M. Oostvogel, Department of Clinical Microbiology, Medical Center Haaglanden, PO Box 432, 2501 CK The Hague, the Netherlands; email: p.oostvogel@mchaaglanden.nl

Catheter-related Bacteremia and Multidrug-resistant *Acinetobacter lwoffii*

To the Editor: *Acinetobacter* species are ubiquitous in the environment. In recent years, some species, particularly *A. baumannii*, have emerged as important nosocomial pathogens because of their persistence in the hospital environment and broad antimicrobial drug resistance patterns (*1*,*2*). They are often associated with clinical illness including bacteremia, pneumonia, meningitis, peritonitis, endocarditis, and infections of the urinary tract and skin (*3*). These conditions are more frequently found in immunocompromised patients, in those admitted to intensive care units, or in those who have intravenous catheters, and those who are receiving mechanical ventilation (*4*,*5*).

The role of *A. baumannii* in nosocomial infections has been documented (*2*), but the clinical effect of other *Acinetobacter* species has not been investigated. *A. lwoffii* (formerly *A. calcoaceticus* var. *lwoffii*) is a commensal organism of human skin, oropharynx, and perineum that shows tropism for urinary tract mucosa (*6*). Few cases of *A. lwoffii* bacteremia have been reported (*3*,*5*–*7*). We report a 4-year (2002–2005) retrospective study of 10 patients with *A. lwoffii* bacteremia admitted to a 600-bed teaching hospital in central Italy.

All 10 patients were immunocompromised; 8 had used an intravascular catheter (peripheral or central) and 2 had used a urinary catheter. Blood cultures of the patients were analyzed with the BacT/ALERT 3D system (bioMérieux, Marcy l'Etoile, France). Isolates were identified as *A. lwoffii* by using the Vitek 2 system and the API 20NE system (both from bioMérieux).

Susceptibilities of 10 *A. lwoffii* isolates to 18 antimicrobial drugs were determined by the broth microdilution method, according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines (*8*). The drugs tested were amikacin, ampicillin-sulbactam, aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, ofloxacin, piperacillin, piperacillin-tazobactam, tetracycline, tobramycin, and trimethoprim-sulfamethoxazole. MIC was defined as the lowest drug concentration that prevented visible bacterial growth. Interpretative criteria for each drug tested were as in CLSI guidelines (*8*). *A. lwoffii* resistant to ≥4 classes of drugs were defined as multidrug-resistant (MDR) isolates.

A. lwoffii isolates were genotyped by pulsed-field gel electrophoresis (PFGE) to determine their epidemiologic relatedness. Chromosomal DNA was digested with *Sma*I (*9*) and analyzed with a CHEF DR II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). PFGE patterns were classified as identical, similar (differed by 1–3 bands), or distinct (differed by ≥4 bands) (*10*).

Among the 10 *A. lwoffii* isolates, 6 were susceptible to all drugs except cephalosporins (cefepime, cefotaxime, ceftazidime, and ceftriaxone) and aztreonam. The other 4 isolates were MDR: 3 were susceptible only to imipenem (MICs 1–4 μg/mL), meropenem (MICs 1–2 μg/mL), and amikacin (MICs 2–4 μg/mL). The fourth MDR strain was susceptible to imipenem (MIC 2 μg/mL), meropenem (MIC 2 μg/mL), amikacin (MIC 4 μg/mL), and ciprofloxacin (MIC 1 μg/mL). Seven antimicrobial drug resistance profiles were detected (Table).

Macrorestriction analysis of the *A. lwoffii* isolates identified 8 distinct PFGE types. Two MDR strains (strains 2 and 3 in the Table), which

Table. Antimicrobial drug susceptibility and pulsed-field gel electrophoresis (PFGE) patterns of 10 *Acinetobacter lwoffii* strains, Italy, 2002–2005

		Drug†																		
No.	Source*	AS	PI	PT	CE	CT	CA	CF	AT	CI	LE	OF	GM	TM	AM	TC	IP	MP	TS	Antibiotype	PFGE
1	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S	R	a	A
2	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S	R	a	B
3	OW	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	S	R	a	B
4	ICU	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	S	S	R	b	C
5	ICU	S	S	R	S	S	R	R	R	R	R	R	S	S	S	S	S	S	S	c	D
6	ICU	R	S	S	S	R	R	R	S	S	S	S	R	R	S	S	S	S	S	d	E
7	UW	S	S	S	R	R	R	R	R	S	S	S	S	S	S	R	S	S	S	e	F
8	ICU	S	R	S	R	R	S	R	R	S	S	S	S	S	S	S	S	S	R	f	G
9	ICU	S	R	S	R	R	S	R	R	S	S	S	S	S	S	S	S	S	R	f	G
10	MW	S	S	S	S	S	R	S	R	S	R	R	R	S	R	S	S	S	S	g	H

*ICU, intensive care unit; OW, orthopedic ward; UW, urologic ward; MW, medical ward.
†AS, ampicillin-sulbactam; PI, piperacillin; PT, piperacillin-tazobactam; CE, cefepime; CT, cefotaxime; CA, ceftazidime; CF, ceftriaxone; AT, aztreonam; CI, ciprofloxacin; LE, levofloxacin; OF, ofloxacin; GM, gentamicin; TM, tobramycin; AM, amikacin; TC, tetracycline; IP, imipenem; MP, meropenem; TS, trimethoprim-sulfamethoxazole; R, resistant; S, susceptible.

were isolated from patients in different wards, and 2 non-MDR strains (strains 8 and 9), which were isolated from patients in the same ward, had similar PFGE patterns and identical resistance phenotypes. These findings suggest nosocomial transmission. Nine of the 10 patients survived after catheter removal or treatment with appropriate antimicrobial drugs. These results confirm that catheter-related *A. lwoffii* bacteremia in immunocompromised hosts is associated with a low risk for death (*4*,*6*).

This study identified *A. lwoffii* MDR strains that cause bacteremia in immunocompromised catheterized patients. Our data are consistent with those of previous reports on the role of catheters as the principal source of *A. lwoffii* infections.

Luciano Tega,* Katia Raieta,† Donatella Ottaviani,‡ Gian Luigi Russo,† Giovanni Blanco,* and Antonio Carraturo*

*Ospedale Santa Maria Goretti, Latina, Italy; †Istituto di Scienze dell'Alimentazione, Avellino, Italy; and ‡Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Ancona, Italy

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Address for correspondence: Antonio Carraturo, Laboratorio di Microbiologia, Ospedale Santa Maria Goretti, Azienda Unità Sanitaria Locale di Latina, Via Canova, 04100 Latina, Italy; email: acarraturo@yahoo.com

Hale Aspacio Woodruff (1900–1980). The Art of the Negro: Interchange (1950–1951) (detail). Oil on canvas (360 cm × 360 cm). Clark Atlanta University Collection of African-American Art, Atlanta, Georgia, USA

Microbiologic and Cultural Interchange

Polyxeni Potter*

"...I did develop…a kind of root down in Atlanta," confided Hale Woodruff during an oral history interview, "You may have heard of it. It was called the 'Outhouse School' and, frankly, it was given such a name as this by one of the press writers because we used to paint landscapes in and around Atlanta in our art classes and the hillsides were just dotted with outdoor toilets" (*1*). The understated and self-effacing Woodruff was referring to the Atlanta School, an alliance he developed among black artists in the 1940s, which flourished into national activities, among them an annual art exhibit. At the inaugural, philosopher Alain Locke, spokesman of the Negro Movement, known in the 1920s and 1930s as Harlem Renaissance, praised the exhibit for encouraging "a healthy and representational art of the people with its roots in its own soil" (*2*).

Painter, muralist, printmaker, experimenter, educator, organizer Woodruff became art director at Atlanta University, where he founded the art department and permanent collection and later painted the Art of the Negro murals. Born in Cairo, Illinois, the only child of a widowed mother, who was "very, very skillful with the pencil and the pen," he grew up in Nashville, Tennessee, and showed early talent as high school newspaper cartoonist and later, during his studies at the John Herron Art Institute in Indianapolis, as weekly political cartoonist for the Indianapolis Ledger (*1*).

Later, Woodruff studied at Harvard University and the Art Institute of Chicago (*3*). He arrived in Atlanta "to paint the red clay of Georgia" by way of Paris, France, where he lived on a shoestring for 4 years, attending the Académie Moderne and Académie Scandinave at the time Ernest Hemingway, F. Scott Fitzgerald, Gertrude Stein, Josephine Baker, Henry O. Tanner, Palmer Hayden, and other American expatriates made Paris their home. Exposure to cubism in Paris guided his transition from realistic scenes of everyday life in the rural South to bold abstraction and invention.

During a summer in Mexico, he studied with Diego Rivera, "I wanted to paint great significant murals in fresco and I went down there to…learn his technique" (*1*). Rivera's murals, which mingled culture, history, and folklore with sociopolitical and communal elements, were cre-

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

ated for public areas, not private galleries. This use of art to reach a broad segment of society appealed to Woodruff (*4*). Inspired by his work in Mexico, he painted the widely acclaimed murals at Talladega College in Alabama, which delineate the development of the college from an abandoned civil war prison and commemorate the uprising on the slave ship Amistad (*5*).

"I've always had a high regard and respect for the African artist and his art…. I look at the African artist certainly as one of my ancestors" (*1*). While Woodruff was studying in Indianapolis, he became friends with German gallery owner and art patron Herman Lieber, who gave him Afrikanische Plastik, a book on African sculpture, which sparked his interest in the subject and indelibly colored his understanding of art. "Then on seeing the work of Paul Cézanne I got the connection. Then I saw the work of Picasso and I saw how Cézanne, Picasso, and the African had a terrific unique sense of form" (*1*).

Woodruff left Atlanta to work at New York University. "I sort of felt that I had done my pioneering down there…" (*1*). His association with Atlanta University, as well as Spelman and Morehouse Colleges, had cemented his teaching career. "It's been my problem and I'm attempting to solve it, to reconcile being a teacher on the one hand and a painter on the other" (*1*). A professor with a full travel schedule of lecturing and exhibiting throughout the United States, Woodruff had to balance practicing art and fulfilling academic duties. But he did not eschew his scholarly work: "So many of the artists who are showing up on Madison Avenue now are people that I've taught at NYU" (*1*).

A thinker as well as painter, he struggled with the demands of social conscience and artistic excellence, trying to define his allegiance as an artist. "I think all art if it's worth its salt has got to be universal. But it comes from a local source, you see. That's it. It can be as local as all getout, but it has to have this transcendental quality in order for it to be universal. Now it can be black art; it can be yellow art; white art; anything. But it comes from a local source" (*1*). He drew on Ralph Ellison, who grappling with the same dilemmas concluded, "I want to be the right arm, the themes of my people, but I want to be a great writer regardless" (*1*).

The Art of the Negro was commissioned after Woodruff moved to New York. The mural, 6 canvas panels (360 cm × 360 cm) in the rotunda of Atlanta University's Trevor Arnett Library, "…has to do with a kind of interpretive treatment of African art….Also, I wanted it to be something of an inspiration to the students who go to that library, to see something about the art of their ancestors" (*1*).

Panel 2, Interchange, on this month's cover, is a dramatic depiction of Woodruff's ideas as well as style. The flat figures, exaggerated forms, and stylized scenes within the larger composition propose a semiabstract version of reality. Woodruff the reader of history and lover of knowledge packed the mural with African, Greco-Roman, and northern European symbols and interlocking scenes of harmonious human interaction, unencumbered by cultural or geographic barriers.

Exchange of knowledge and ideas is at the heart of both the interaction and the symbols that preceded it. For human achievement is multiethnic and multicultural, formed of infinite exchanges, intentional, as well as imperceptible and unacknowledged. Woodruff's clear vision of an interconnected, reciprocal, and multifaceted world has broad application in what we now know is also a microbiologic interchange, just as crucial to human achievement and survival.

Whether straightforward as travel in the spread of African tickbite fever, unexpected and insidious as a link between malaria and HIV, zoonotic as the transmission of many emerging infections, or borne of human effort as bednet use to prevent mosquitoborne disease, interchange is key to science, as well as art.

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the March issue for the following topics:

Bird Migration Routes and Risk for Pathogen Dispersion into Western Mediterranean Wetlands

Human and Animal Vaccination Delivery to Remote Nomadic Families, Chad

Global Survey of Second-Line Drug Resistance among *Mycobacterium tuberculosis* Isolates and Emergence of Extensively Drug-resistant Tuberculosis

Behavioral Risks for West Nile Virus Disease, Northern Colorado, 2003

Matrix Protein 2 Vaccination and Protection against Influenza Viruses, Including H5N1

Multiyear Surveillance of Influenza Virus Type A in Migratory Waterfowl in Northern Europe

Tandem Repeat Analysis for Surveillance of Human *Salmonella* Typhimurium Infections Analysis

Diversity and Distribution of the Relapsing Fever Spirochete *Borrelia hermsii*, Western North America

In Vitro Cell Culture Infectivity Assay for Human Noroviruses

Laboratory Surveillance for AmpC β-Lactamase-producing *Escherichia coli*, Calgary

Risk for Infection with Highly Pathogenic Influenza A (H5N1) Virus in Chickens, Hong Kong, 2002

Complete list of articles in the March issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 23–25, 2007
IMED 2007: International Meeting on Emerging Diseases and Surveillance
Vienna, Austria
Contact: info@isid.org or
617-277-0551 voice
617-278-9113 fax
http://imed.isid.org

February 27–March 2, 2007
5th Annual American Society for Microbiology (ASM) Biodefense and Emerging Diseases Research Meeting
Renaissance
Washington, DC, USA
http://www.asmbiodefense.org
7200.asp

March 7–9, 2007
6th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2007)
Raffles City Convention Centre
Singapore
http://www.isaar.org

March 20–23, 2007
ISOPOL XVI: 16th International Symposium on Problems of Listeriosis
Marriott Riverfront Hotel
Savannah, GA, USA
Contact: 240-485-2776
http://www.aphl.org/conferences/ISOPOL.cfm

April 30–May 2, 2007
10th Annual Conference on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org

Announcement Submission

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a web site that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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University of British Columbia Museum of Anthropology, Vancouver, British Columbia, Canada. Photo: Bill McLennan

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A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.