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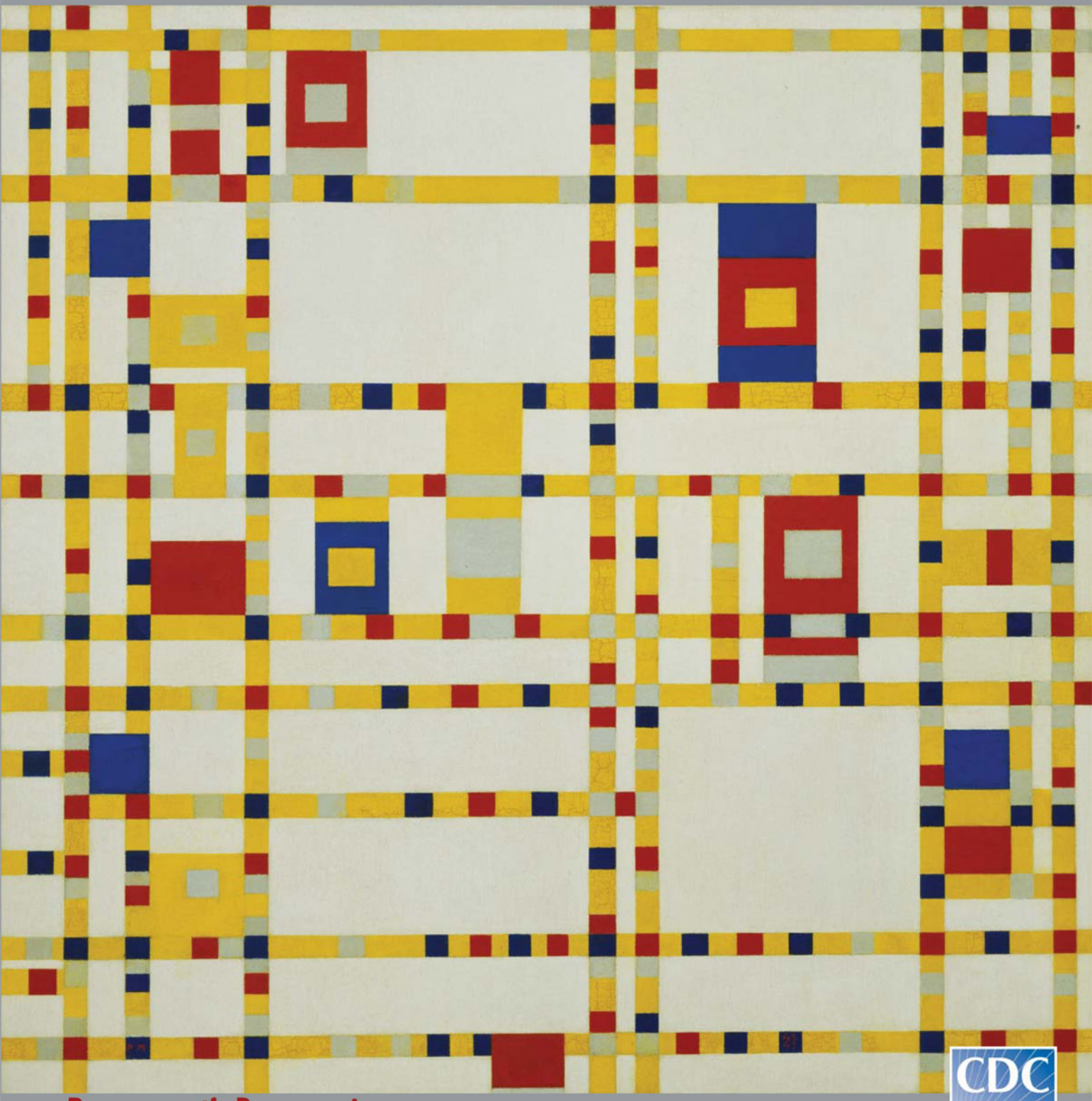
Vol.10, No.10, October 2004



Pneumocystis Pneumonia

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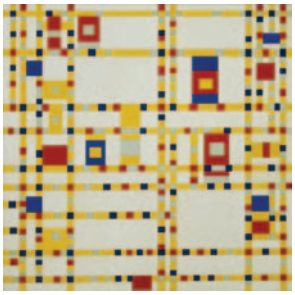

On the Cover

Piet Mondrian (1872–1944).
Broadway Boogie Woogie (1942–1943).
Oil on canvas, 127 cm x 127 cm. The Museum of Modern Art, New York, NY, USA
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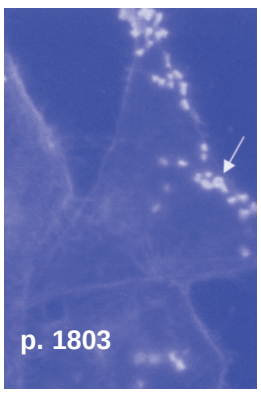

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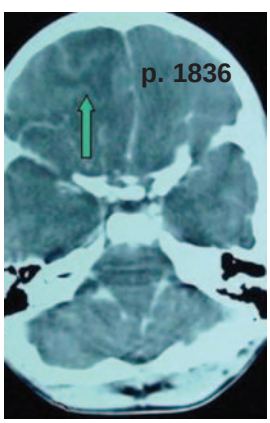

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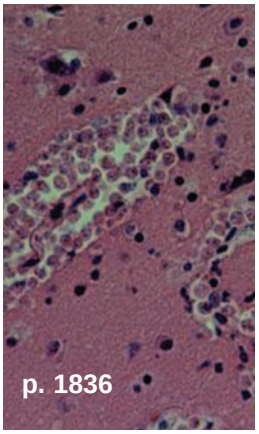

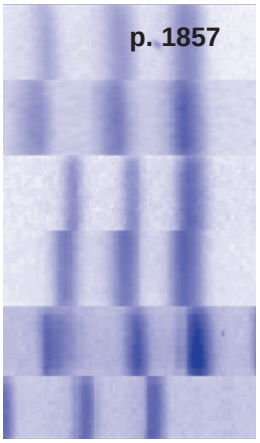

Letters

News and Notes

Current Epidemiology of *Pneumocystis* Pneumonia

Alison Morris,*† Jens D. Lundgren,‡ Henry Masur,§ Peter D. Walzer,¶ Debra L. Hanson,# Toni Frederick,# Laurence Huang, Charles B. Beard,†† and Jonathan E. Kaplan#**

Pneumocystis pneumonia (PCP) has historically been one of the leading causes of disease among persons with AIDS. The introduction of highly active antiretroviral therapy in industrialized nations has brought about dramatic declines in the incidence of AIDS-associated complications, including PCP. In the adult population, the incidence of PCP has significantly decreased, but it remains among the most common AIDS-defining infections. Similar declines have been documented in the pediatric population. In much of the developing world, PCP remains a significant health problem, although its incidence among adults in sub-Saharan Africa has been debated. This review discusses the epidemiology of PCP during the current era of the AIDS epidemic. Although fewer cases of PCP occur in industrialized countries, increasing drug-resistant HIV infections, possible drug-resistant PCP, and the tremendous number of AIDS cases in developing countries make this disease of continued public health importance.

Pneumocystis pneumonia (PCP), which is caused by *Pneumocystis jirovecii* (formerly *P. carinii* f. sp. *hominis*), is frequently the first serious illness encountered by HIV-infected persons. During the early years of the AIDS epidemic, PCP was the AIDS-defining illness for as many as two thirds of patients in the United States. Although a decline in incidence of PCP occurred during the era of highly active antiretroviral therapy (HAART), PCP remains the most common serious opportunistic illness in HIV-infected persons (1). Patients in the developing world without access to PCP prophylaxis or antiretroviral drugs remain at high risk, and PCP continues to develop in certain groups in industrialized countries.

The drug of choice for treatment and chemoprophylaxis of PCP is trimethoprim-sulfamethoxazole (TMP-SMX). In recent years, antimicrobial drug resistance has emerged as a possible cause of failure of patients to respond to TMP-SMX. Investigators have demonstrated an association between exposure to sulfa drugs and mutations in the dihydropteroate synthase (DHPS) gene of *P. jirovecii*, but the relationship between these mutations and treatment (or prophylaxis) failure is unclear. Understanding whether DHPS mutations cause antimicrobial drug resistance is important in guiding clinicians who care for patients with PCP.

A series of articles in this issue of Emerging Infectious Diseases highlights the continuing importance of PCP, the potential for drug resistance, and laboratory techniques that can be used to study the problem. We hope that these articles will stimulate interest in exploring the relationship between DHPS mutations and resistance of *P. jirovecii* to sulfa-containing drugs and in assessing DHPS mutations as possible causes of treatment failure in patients with PCP. In this introductory article, we summarize the changes in incidence of PCP since the introduction of HAART, discuss groups at risk for PCP in developing and industrialized nations, and examine possible future trends in the disease. A data collection form has been included online with this series of articles to assist in the collection of appropriate and standardized data from patients with PCP and to facilitate comparing and pooling data from different centers (online Appendix available from http://www.cdc.gov/ncidod/eid/vol10no10/03-0985_ app.pdf).

PCP before HAART

The first clinical cases of PCP were reported during World War II in orphanages in Europe. These cases of "plasma cell pneumonia" were common among malnourished children and were later reported in children in

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Iranian orphanages. The disease was then recognized in patients who were immunocompromised because of malignancies, immunosuppressive therapy, or congenital immunodeficiencies. Solid organ transplantation increased the number of patients at risk for PCP, although rates diminished after chemoprophylaxis was introduced. Without chemoprophylaxis, rates of PCP are 5%–25% in transplant patients, 2%–6% in patients with collagen vascular disease, and 1%–25% in patients with cancer. Defects in CD4+ lymphocytes are a primary risk factor for developing PCP, but the immune response to *Pneumocystis* is complex. CD8+ lymphocytes seem to be important in *Pneumocystis* clearance, and defects in B-cells and antibody production may also predispose to PCP.

The beginning of the AIDS epidemic in the early 1980s shifted the incidence of PCP from a rare disease to a more common pneumonia. Clusters of PCP cases in homosexual men and intravenous drug users were one of the first indications of the HIV epidemic (2). PCP rapidly became the leading AIDS-defining diagnosis in HIV-infected patients. In the initial stages of the epidemic, PCP rates were as high as 20 per 100 person-years for those with CD4+ cell counts <200 cells/μL (3). PCP was responsible for two thirds of AIDS-defining illnesses, and an estimated 75% of HIV-infected patients would develop PCP during their lifetime (4).

The first substantial decline in the incidence of PCP occurred after the introduction of anti-*Pneumocystis* prophylaxis in 1989 (5). Although absolute numbers of cases of PCP as an AIDS-defining illness in the United States remained stable from 1989 to 1992 because of an increasing incidence of AIDS, the percentage of AIDS cases with PCP declined from 53% in 1989 to 49%, 46%, and 42% in 1990, 1991, and 1992, respectively (Centers for Disease Control and Prevention, AIDS Surveillance Summaries, 1989–1992). The later use of combination antiretroviral therapy further reduced the rates of PCP among adults by 3.4% per year after 1992 (1).

PCP in Adults in Industrialized Countries after HAART

Incidence

The advent of HAART has resulted in further declines in rates of PCP and other opportunistic infections (1). Several large, multicenter studies have specifically tracked the incidence and epidemiologic features of PCP. The largest is the Adult and Adolescent Spectrum of HIV Disease (ASD) Project. Data from this project indicated a marked reduction in the incidence of all opportunistic infections in 1996 and 1997, when HAART first became widely available (Figure 1). PCP cases decreased 3.4% per year from 1992 through 1995; the rate of decline of PCP increased to 21.5% per year from 1996 through 1998 (1). Despite this improvement, PCP is still the most common AIDS-defining opportunistic infection in the United States.

The Multicenter AIDS Cohort study (MACS) has followed >5,000 homosexual men since 1984 (6). Of these, 2,195 were either HIV-infected at time of enrollment or seroconverted to HIV during the study. Opportunistic infection rates were compared for the HAART era (1996–1998) and the era of antiretroviral monotherapy (1990–1992) (7). For persons who seroconverted during the study period, the relative hazard for development of PCP from seroconversion to initial AIDS-defining opportunistic infection was 0.06 during the HAART era compared to the time of monotherapy. For those already diagnosed with AIDS, the study found a hazard of 0.16, which demonstrated a dramatically lower risk for PCP during the HAART era.

In Europe, the EuroSIDA study has followed a cohort of >8,500 HIV-infected patients. The investigators examined changes in incidence of AIDS-defining illnesses before and after HAART was introduced and found results similar to those in North America (8). PCP cases decreased over time (1994–1998). Incidence of PCP fell from 4.9 cases per 100 person-years before March 1995 to 0.3 cases per 100 person-years after March 1998 (9).

Occurrence in Relation to PCP Prophylaxis

PCP still occurs in industrialized nations despite the availability of HAART and anti-*Pneumocystis* prophylaxis.

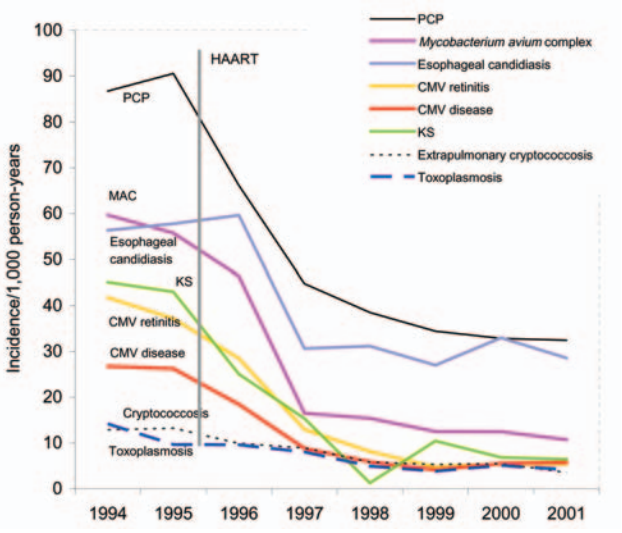

Figure 1. Yearly opportunistic infection rates per 1,000 person-years, CDC Adult and Adolescent Spectrum of Disease Project, 1994–2001. CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; KS, Kaposi's sarcoma; MAC, *Mycobacterium avium* complex; PCP, *Pneumocystis* pneumonia. Data are standardized to the population of AIDS cases reported nationally in the same year by age, sex, race, HIV exposure mode, country of origin, and CD4+ lymphocyte count.

ASD investigated the history of prescriptions for PCP prophylaxis in HIV-infected adults in whom developed PCP from 1999 through 2001 (Figure 2). Almost 44% of PCP cases occurred in patients not receiving medical care, most of whom were probably not known to be HIV-infected. Forty-one percent of patients were prescribed prophylaxis but did not adhere to treatment, or PCP developed despite their taking medications appropriately. Possible explanations for PCP in the "breakthrough" group include the development of drug-resistant *Pneumocystis* or decreased efficacy of prophylaxis in those with low CD4+ cell counts. An additional 9.6% of patients were under medical care and should have received prophylaxis based on current recommendations, but had not been prescribed prophylaxis by their providers. Five percent of patients were under care but did not meet criteria for prophylaxis.

Risk Factors

A CD4+ cell count <200 cells/μL was the leading pre-HAART risk factor for PCP and remains an important risk factor in the HAART era. The risk for PCP increases exponentially the lower the CD4+ cell count is below 200 cells/μL (10). When patients on HAART have sustained increases in CD4+ cell counts >200 cells/μL, the risk for PCP decreases sufficiently to safely discontinue both primary and secondary prophylaxis (9,11). Those in whom PCP develops while on HAART typically have low CD4+ cell levels. ASD found that the median CD4+ cell count in persons with PCP while on HAART was extremely low (29 cells/μL), although the count was somewhat higher than for those not on HAART (13 cells/μL) (1). The EuroSIDA study reported that persons on HAART in whom PCP developed had a median CD4+ cell count of 30 cells/μL, identical to those with PCP who were not receiving HAART (8). Patients without improvement in their CD4+ cell count despite use of HAART remain at risk for PCP, and PCP still rarely occurs in persons with CD4+ cell counts >200 cells/μL.

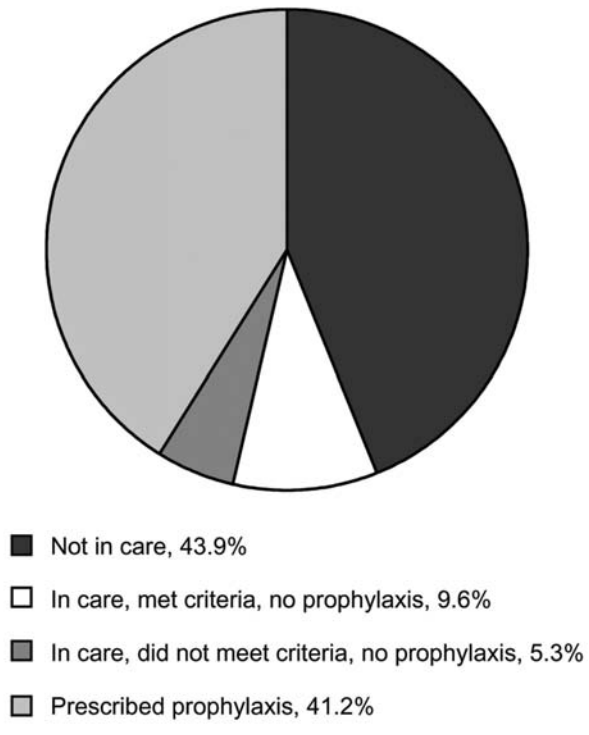

Figure 2. Classification of *Pneumocystis* pneumonia cases from 1999–2001, CDC Adult and Adolescent Spectrum of HIV Disease Project, n = 1,073.

Other clinical factors such as sex, race or ethnicity, and HIV transmission category have been examined as risk factors for PCP. Men and women appear to have an equivalent risk for PCP (12). One study demonstrated that African Americans have approximately one third the risk for PCP as white persons (10), but this finding has not been replicated (12). PCP risk according to HIV transmission category is also debated. One autopsy study found that PCP was less common in intravenous drug users than in other risk groups (13). Kaplan et al. found a slightly increased risk for those men who had sex with men and were intravenous drug users, but risk was equivalent in other transmission categories (12).

Risk for *Pneumocystis* Colonization

Although PCP cases have declined, polymerase chain reaction (PCR) has led to the discovery of *Pneumocystis* DNA in asymptomatic persons. *Pneumocystis* in respiratory specimens from persons who do not have signs or symptoms of clinical infection and who do not progress to infection has been defined as colonization or subclinical carriage. Often, *Pneumocystis* DNA is detected only by PCR, and the organism is not seen on routine histochemical staining. The clinical significance of *Pneumocystis* in respiratory specimens and the viability of organisms detected only by PCR are unknown. However, colonization may be important for several reasons. *Pneumocystis* colonization may increase the risk for progression to PCP, carriers of the organism may transmit infection to others, and latent infection may lead to inflammation that is detrimental to the lung. Most healthy persons do not have detectable *Pneumocystis* in respiratory specimens, but rates of colonization may be as high as 69% in HIV-infected persons (14). Recent evidence suggests that non–HIV-infected persons may also be colonized with *Pneumocystis*, thus increasing the potential number of persons affected (15).

PCP in Children in Industrialized Countries

Incidence

Early in the HIV epidemic, PCP occurred in HIV-infected children at a rate of 1.3 cases per 100 child-years

from infancy to adolescence and was as high as 9.5 cases per 100 child-years in the first year of life (16,17). In the 1990s, pediatric HIV infection decreased, primarily as a result of improved prenatal HIV testing and use of HIV treatment to prevent vertical transmission of the virus. The Pediatric Spectrum of Disease (PSD) study found significant decreases in the rates of most opportunistic infections in HIV-infected children during the HAART era (Figure 3). PCP cases declined significantly from 1992 to 1997, with an increase in the rate of decline after 1995, presumably from HAART (1). Because widespread use of HAART for children has occurred more recently than for adults, the full effect of HAART on pediatric PCP likely has not yet been realized.

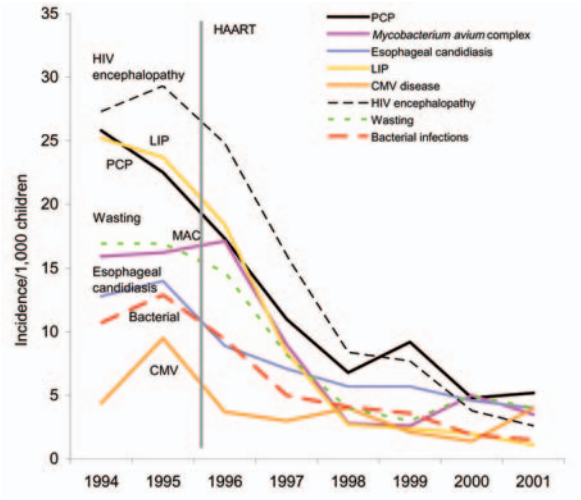

Figure 3. Yearly opportunistic infection rates per 1,000 HIV-infected children, CDC Pediatric Spectrum of Disease Project, 1994–2001. Bacterial, bacterial infections; CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; LIP, lymphocytic interstitial pneumonia; MAC, *Mycobacterium avium* complex; PCP, *Pneumocystis* pneumonia. Incidence rates were calculated per 1,000 children at risk each year. All trends were significant at $p < 0.05$ in chi-square for trend analysis for four age groups (<1 year, 1–5 years, 6–9 years, and >10 years) except for the <1-year-old group for PCP, bacterial, and MAC.

Risk Factors

The occurrence of PCP in infants does not seem to be related to the CD4+ cell count in the same manner as in adults, although it is related to the percentage of CD4+ cells and CD4+ cell counts are below normal in children <1 year of age with PCP (18). Furthermore, peak incidence of PCP occurs in infants 3–6 months of age, when HIV status may still be undetermined. Implementing recommendations to initiate PCP prophylaxis in all infants born to HIV-positive mothers decreased the incidence of the disease in the pediatric population before the advent of HAART (11). For children older than 6 years of age, the CD4+ cell count predicts disease in a manner similar to adults, and CD4+ cell counts <200 cells/μL are still considered an indication for prophylaxis (11).

Although HAART decreased the incidence of PCP in children, it has not eliminated the disease, mostly because of failure to identify HIV-infected mothers. PCP seems to occur early in life among HIV-infected infants, which suggests that exposure to *Pneumocystis* is common. In fact, anti-*Pneumocystis* antibodies develop in most nonimmunocompromised children in the first several years of life (19,20). A British study of children with PCP in the beginning of the HAART era found that PCP developed in 83 of 531 children with perinatally acquired HIV as their first AIDS indicator disease, which represented ≈50% of AIDS diagnoses (21). Most of these children were <12 months of age, and 79% were born to mothers not previously diagnosed with HIV. Given that the mothers had unrecognized HIV disease, HAART would be expected to have little effect on disease incidence in this population, and improved maternal screening would be more important for disease prevention.

Risk for *Pneumocystis* Colonization

Colonization may occur at higher rates in healthy children than in healthy adults. Vargas recently documented that nested PCR was positive for *Pneumocystis* DNA in the nasopharyngeal aspirates of 32% of 74 healthy Chilean infants (20). Children dying of sudden infant death syndrome (SIDS) also have a high rate of *Pneumocystis* (51 [30%] of 171), as seen on microscopy of lung specimens (22). The role *Pneumocystis* plays in SIDS is not understood. Similar to the adult population, effects of *Pneumocystis* colonization and relationship to PCP developing or transmission of infection are not known.

PCP in the Developing World

In contrast to the dramatic improvements in the industrialized world, persons living in developing nations continue to be devastated by HIV. The World Health Organization estimates that 42 million persons were living with HIV at the end of 2002 and that 95% of these persons live in developing countries. Large portions of the populations of Southeast Asia and sub-Saharan Africa are infected with HIV, and an estimated 55 million persons will die of AIDS in sub-Saharan Africa from 2000 through 2020. HIV is also increasing in areas such as Latin America, eastern Europe, and Asia. Despite increasing efforts to supply affordable therapy to these nations, HAART is not widely available.

PCP still occurs frequently in many parts of the developing world (23). Studies from Thailand show a prevalence of 27% to 40% among HIV-infected patients treated at a university hospital clinic (24,25). Central and South

Table 1. Summary of selected studies of *Pneumocystis* pneumonia in African adults[a]

Study (ref)	Site	Study period	No. patients	Population	Diagnostic sample/method	% with PCP (n)
Abouya, 1992 (30)	Cote d'Ivoire	1989	53	Died while inpatient	Autopsy lung tissue/Grocott	8 (4)
Ansari, 2002 (31)	Botswana	1997–1998	104	Died while inpatient	Autopsy lung tissue/Grocott	11 (11)
Aderaye, 2003 (32)	Ethiopia	1996	119	Symptomatic, AFB negative, outpatients	Expectorated sputum/IF	11 (13)
Batungwanayo, 1994 (33)	Rwanda	1990	111	Symptomatic, AFB negative, outpatients	BAL, Tbbx/Methenamine silver	5 (5)
Kamanfu, 1993 (34)	Burundi	1991	222	Symptomatic, hospitalized	BAL/Giemsa, IF	5 (11)
Malin, 1995 (35)	Zimbabwe	1992–1993	64	Symptomatic, hospitalized, AFB negative	BAL/Methenamine silver, diff-quik, toluidine blue-O	33 (21)
Worodria, 2003 (36)	Uganda	1999–2000	83	Symptomatic, hospitalized, AFB negative	BAL/IF	39 (32)

[a]AFB, acid-fast bacilli; BAL, bronchoalveolar lavage; IF, immunofluorescence; IS, induced sputum; NPA, nasopharyngeal aspirate.

America also have a large number of PCP cases. One Brazilian study found that 55% of HIV-infected persons with respiratory complaints were diagnosed with PCP, although a small autopsy study of hospitalized Brazilian patients found only 13% to have PCP (26,27). Other studies in this region report PCP prevalence from 24% to 29%, depending on the population studied (28,29).

PCP in Adults in Africa

In contrast to the situation in many other developing regions, PCP has been thought to be rare in African adults. Several representative series are summarized in Table 1. Most early studies reported prevalence rates of 0% to 11% in HIV-infected patients (30,31,33,34), although one early study found a rate of 33% (35).

PCP might not have been commonly reported in Africa for several reasons. Limited resources for diagnosis may have led to lower estimates of PCP. Experienced laboratory personnel are required to prepare and interpret diagnostic specimens. Bronchoscopy is expensive, and induced sputum also requires specialized equipment and personnel to obtain adequate samples. Limited resources make empiric therapy of HIV-infected persons with pneumonia common, possibly leading to inaccurate estimates of the true incidence of PCP. HIV-infected African adults also have high rates of bacterial pneumonia and tuberculosis, diseases that may result in death at higher CD4+ cell counts and prevent many HIV-infected patients from reaching a stage at which they would be susceptible to PCP. Environmental factors, such as seasonal variations, might contribute to a low rate of PCP in Africa. However, high rates of anti-*Pneumocystis* antibodies among African children suggest that exposure to the organism is common (19). Regional strains may be less virulent, or the population may be more resistant, as HIV-infected African Americans have been shown to have lower PCP rates compared to white Americans (10). Detailed molecular study of the organism in different parts of the world is needed to resolve these issues.

The incidence of PCP in Africa may be growing as the AIDS epidemic progresses. A recent review concluded that cases of PCP seem to have increased over time (23), but whether this increase resulted from actual changes in PCP incidence or from improved detection techniques is unclear. Some studies have reported higher rates of PCP in Africa compared to past findings (Table 1). Malin et al. studied a group of 64 hospitalized HIV-infected patients in Zimbabwe in 1995 (35). These patients had pneumonia unresponsive to penicillin, and sputum samples were smear-negative for acid-fast bacilli (AFB). All patients underwent bronchoscopy with bronchoalveolar lavage (BAL). Twenty-one (33%) of these patients had PCP. Reasons for a higher rate of PCP among these patients included use of definitive diagnosis and probable selection bias by including only patients with severe pneumonia when other diagnoses, such as tuberculosis, had been excluded. Another study examined 83 patients hospitalized with respiratory symptoms (36). All patients had sputum cultures that were negative for AFB and underwent bronchoscopy with BAL for diagnosis. Thirty-two patients (38.6%) were diagnosed with PCP. Not all studies have found high rates of PCP. Aderaye et al. reported that of 119 outpatients with respiratory symptoms and negative AFB cultures, only 11% had PCP (32). Similarly, another recent study found PCP in 11% of patients who underwent autopsy after dying as an inpatient with respiratory symptoms (31). Future research will be needed to clarify the risk for PCP in Africa.

PCP in Children in Africa

In contrast to adults, HIV-infected children in Africa have high rates of PCP. Autopsy series describe rates of

PCP from 14% to 51.3%, depending on the age group studied (Table 2). Ikeogu et al. found that in Zimbabwe, 19 (15.5%) of 122 HIV-infected children who died <5 years of age had evidence of PCP at autopsy (40). All cases except one were in infants <6 months. Another autopsy study from the early 1990s found that PCP was present in 11 (31%) of 36 HIV-infected infants but was not found in 42 HIV-infected children >15 months (41). The largest autopsy series examined 180 HIV-infected children in Zambia (38). Twenty-nine percent of the children died of PCP, making PCP the third leading cause of death overall. Among children <6 months of age, PCP was the most common cause of pneumonia, detected in 51.3%. Six of 84 HIV-negative children had evidence of PCP at autopsy. The most recent autopsy series reported that 10 (28.6%) of 35 HIV-infected children had PCP (37).

Because autopsy studies examine terminal disease, their assessment of disease prevalence might be biased. Several authors described prevalence of PCP among children in clinic or hospital settings to estimate disease frequency more accurately. Most studies reported rates higher than those in adults. Two authors found rates >40% among HIV-infected children hospitalized with pneumonia (42,43). Ruffini studied children from 2 to 24 months of age with pneumonia and found that 48.6% had PCP (43). Madhi found that in 231 episodes of pneumonia in HIV-infected children, 101 (43.7%) were due to PCP (39). PCP was most common in infants <6 months, although 35.7% of pneumonias in older children were also caused by PCP. Graham, in a smaller study of 16 cases of PCP in 93 children with HIV infection, also found that most cases of PCP occurred in infants (42). The study reporting the lowest frequency of PCP among children with pneumonia found 15 (9.9%) of 151 HIV-infected children to have PCP (44). Four non–HIV-infected children also had PCP. The authors speculated that the lower rate of PCP in their study may have been attributable to their inability to follow negative sputum examinations with bronchoscopy.

The Future of PCP

The decline in PCP incidence in the industrialized world may be short-lived. Although current regimens are effective in treating HIV, as many as 19% of patients starting HAART will have a viral level >10,000 copies/mL after 48 weeks of treatment (45). In the EuroSIDA cohort, an increasing proportion of HIV-infected patients have been exposed to all classes of antiretrovirals, with 47% of their cohort exposed to nucleoside reverse transcriptase inhibitors, protease inhibitors, and non-nucleoside reverse transcriptase inhibitors by 2001 (45). Of those patients in the cohort with multidrug-resistant HIV who received salvage regimens, a new AIDS-defining opportunistic infection developed in 11%. Growing transmission of resistant HIV is also likely. If new drugs do not become available, the number of patients with resistant virus and opportunistic infections, including PCP, will continue to climb.

Not only is HIV developing resistance, but *Pneumocystis* may also develop resistance to standard prophylaxis and treatment regimens. Many researchers have reported mutations of *Pneumocystis* in response to use of sulfa- or sulfone-containing anti-*Pneumocystis* regimens. Whether these mutations increase the likelihood of prophylaxis or treatment failure is unclear and is reviewed in other papers in this series.

Conclusion

Despite the declines in death and disease from HIV in the United States and western Europe, PCP remains an

Table 2. Summary of selected studies of *Pneumocystis* pneumonia in African children[a]

Study (ref)	Site	Study period	No. patients	Population/age	Diagnostic sample/method	% with PCP (n)
Ansari, 2003 (37)	Botswana	1997–1998	35	Died while inpatient/ 1–13 y	Autopsy lung tissue/Grocott	29 (10)
Chintu, 2002 (38)	Zambia	1997–2000	180	Died from respiratory disease/1 mo–16 y	Autopsy lung tissue/ Methenamine silver	29 (52)
Graham, 2000 (39)	Malawi	1996	93	Hospitalized for pneumonia/2 mo–5 y	NPA/IF	17 (16)
Ikeogu, 1997 (40)	Zimbabwe	1992–1993	122	Died on arrival to hospital/<5 y	Autopsy lung tissue/ Methenamine silver	16 (19)
Lucas, 2000 (41)	Côte d'Ivoire	1991–1992	78	HIV-positive undergoing autopsy, inpatient or outpatient/1 mo–12 y	Autopsy lung tissue/unknown	14 (11)
Madhi, 2002 (42)	South Africa	2000–2001	185 (231 episodes)	Hospitalized with severe pneumonia/1–38 mo	IS, NPA/IF	44 (101)
Ruffini, 2002 (43)	South Africa	1999	105	Hospitalized with severe pneumonia/2–24 mo	IS, NPA/IF	49 (51)
Zar, 2001 (44)	South Africa	1998	151	Hospitalized with pneumonia/3–16 mo	BAL, IS, NPA/ Methenamine silver, IF	10 (15)

[a]BAL, bronchoalveolar lavage; IF, immunofluorescence; IS, induced sputum; NPA, nasopharyngeal aspirate.

important disease and is unlikely to be eradicated. In industrialized nations, PCP still occurs in those not yet diagnosed with HIV or not in medical care, those not receiving PCP prophylaxis, and those not taking or not responding to HAART. Resistance in HIV and *Pneumocystis* may contribute to future increases in PCP incidence. In most developing nations, AIDS patients are at high risk for PCP. In sub-Saharan Africa, the effect of disease from PCP in infants and children is high and is probably greater in adults than previously recognized. Colonization rates among both HIV-infected and non–HIV-infected populations may also be substantial. Better understanding of the epidemiology and transmission of PCP and improved efforts in prevention and treatment are needed.

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Dihydropteroate Synthase Gene Mutations in *Pneumocystis* and Sulfa Resistance

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Pneumocystis pneumonia (PCP) remains a major cause of illness and death in HIV-infected persons. Sulfa drugs, trimethoprim-sulfamethoxazole (TMP-SMX), and dapsone are mainstays of PCP treatment and prophylaxis. While prophylaxis has reduced the incidence of PCP, its use has raised concerns about development of resistant organisms. The inability to culture human *Pneumocystis*, *Pneumocystis jirovecii*, in a standardized culture system prevents routine susceptibility testing and detection of drug resistance. In other microorganisms, sulfa drug resistance has resulted from specific point mutations in the dihydropteroate synthase (DHPS) gene. Similar mutations have been observed in *P. jirovecii*. Studies have consistently demonstrated a significant association between the use of sulfa drugs for PCP prophylaxis and DHPS gene mutations. Whether these mutations confer resistance to TMP-SMX or dapsone plus trimethoprim for PCP treatment remains unclear. We review studies of DHPS mutations in *P. jirovecii* and summarize the evidence for resistance to sulfamethoxazole and dapsone.

Although decreasing in incidence as a result of combination antiretroviral therapy and effective prophylaxis, *Pneumocystis* pneumonia (PCP), caused by *Pneumocystis jirovecii* (formerly *P. carinii* f. sp. *hominis*), remains the most common AIDS-defining opportunistic infection, as well as the most frequent serious opportunistic infection in HIV-infected persons, in the United States and Europe. Despite the fact that this infection can be prevented, certain patients continue to be at increased risk for PCP. Specifically, PCP frequently signals HIV infection in patients not previously known to be HIV-infected (1). Patients who are not receiving regular medical care, as well as those who are not receiving or responding to antiretroviral therapy or prophylaxis, are also at increased risk for PCP (2). PCP may also develop in other immunosuppressed populations, such as cancer patients and transplant recipients. Furthermore, PCP remains a leading cause of death among critically ill patients, despite advances in treatment and management (3).

The first-line treatment and prophylaxis regimen for PCP is trimethoprim-sulfamethoxazole (TMP-SMX) (4). While prophylaxis has been shown to reduce the incidence of PCP, the widespread and long-term use of TMP-SMX in HIV patients has raised concerns regarding the development of resistant organisms. Even short-term exposure to TMP-SMX can be associated with the emergence of TMP-SMX resistance, as has been demonstrated in patients with acute cystitis caused by *Escherichia coli* (5). Indeed, an increased number of sulfa-resistant bacteria have been isolated in HIV patients, which coincides with the rise in TMP-SMX prophylaxis for PCP (6,7). In one study, the prevalence of TMP-SMX–resistant *Staphylococcus aureus* and *Enterobacteriaceae* species isolated in all hospitalized patients increased significantly from <5.5% of isolates before 1986 to 20% in 1995, during which time TMP-SMX prophylaxis was increasing in HIV-infected patients (6). In addition, the rise in resistant organisms was significantly more prominent in samples obtained from HIV-infected patients, in whom resistant isolates increased from 6.3% in 1988 to 53% in 1995. Another study found that significantly more TMP-SMX–resistant organisms were isolated from HIV-infected patients who had received TMP-SMX than from patients who had not received TMP-SMX (7).

Given the emergence of resistance to TMP-SMX among many bacteria (8), concern has focused on the potential development of resistant *Pneumocystis*. Based

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on animal studies, nearly all of the anti-*Pneumocystis* activity of TMP-SMX is due to sulfamethoxazole (9). The development of sulfonamide resistance could result in the failure of sulfamethoxazole as well as dapsone, a sulfone antimicrobial agent also used in the treatment and prophylaxis of PCP. While separate lines of investigation also suggest that *Pneumocystis* may be developing resistance to atovaquone, a second-line PCP treatment and prophylaxis regimen (10), we concentrate our review on the evidence for the development of sulfonamide-resistant *Pneumocystis*.

Mechanisms of Sulfonamide Resistance

Sulfonamides act by interfering with folate synthesis. Since many microorganisms cannot transport folate into cells as mammalian cells can, most prokaryotes and lower eukaryotes must synthesize folates de novo (11). Sulfonamides inhibit one of the integral enzymes in folate synthesis, dihydropteroate synthase (DHPS), which catalyzes the condensation of para-aminobenzoic acid and pteridine to form dihydropteroic acid (Figure). Since mammalian cells lack DHPS, sulfonamides can selectively inhibit the growth of various microorganisms. Trimethoprim, part of the fixed combination TMP-SMX, inhibits another of the integral enzymes, the dihydrofolate reductase (DHFR).

Resistance to sulfonamides can emerge by means of a number of mechanisms (12). In most gram-negative enteric bacteria, sulfonamide resistance is largely plasmid-borne and related to drug-resistant DHPS variants with substantial sequence divergence (12). Chromosomal mutations in the DHPS locus—such as point mutations, insertions of duplicate amino acids, or larger sequence alterations as a result of recombination—can also lead to resistance (8). In some organisms, several different mechanisms of resistance have been identified in different strains. For example, some strains of *Neisseria meningitidis* have acquired a DHPS gene with 10% sequence divergence, postulated by others to be due to recombination (12), whereas other *Neisseria* strains have acquired a chromosomal insertion, resulting in the addition of two amino acids to DHPS (13). In other organisms, such as *E. coli* and *Plasmodium falciparum*, nonsynonymous point mutations resulting in amino acid substitutions in DHPS can confer sulfa resistance (14,15). Furthermore, the accumulation of additional mutations over time can confer increasing levels of sulfa resistance, as has occurred in *P. falciparum* (16).

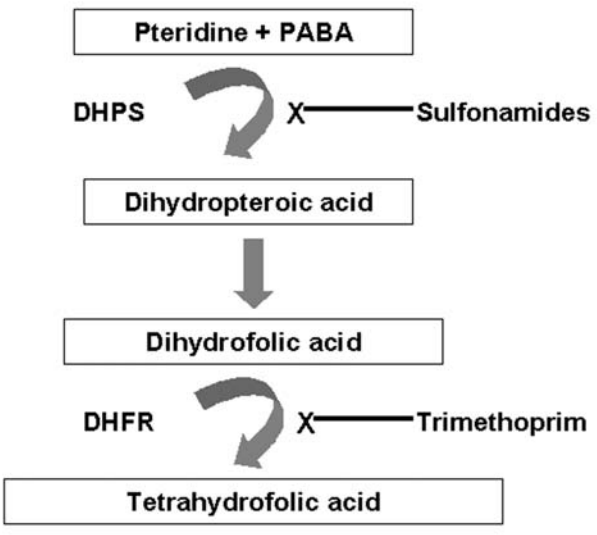

Figure. Inhibition of folate synthesis by sulfonamides and trimethoprim. PABA, paraaminobenzoic acid; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase.

Dihydropteroate Synthase Mutations in *Pneumocystis*

Similar to other microorganisms, mutations have been identified in the DHPS gene of *Pneumocystis jirovecii*, which has raised the question of whether *P. jirovecii* is developing resistance to sulfonamides. The DHPS gene of *P. jirovecii* has been sequenced and is part of the folic acid synthesis gene or *fas* gene; it encodes a trifunctional protein along with dihydroneopterin aldolase and hydroxymethyldihydropterin pyrophosphokinase (17). Sulfa medications appear to exert selective pressure on *Pneumocystis* (18), as the DHPS gene is more likely to display mutations in highly conserved regions in patients with PCP who have previously been exposed to sulfa medications (19–25). These DHPS gene mutations were rarely found in clinical isolates before the early 1990s (19,20,22). Genetic analysis suggests that the mutations arose independently in multiple strains of *Pneumocystis*, which supports the theory that exposure to sulfa medications selects for DHPS gene mutations (26). Furthermore, DHPS gene mutations have not been found in other mammalian *Pneumocystis* species that have not been exposed to sulfa medications (18,27).

Several factors suggest that the mutations observed in *P. jirovecii* may confer resistance to sulfa medications. The region of the DHPS gene in which mutations have been identified is one that is highly conserved among other organisms, including *Plasmodium falciparum*, *Streptococcus pneumoniae*, *E. coli*, and *Bacillus subtilis* (18). The most common mutations identified in the *Pneumocystis jirovecii* DHPS are nonsynonymous point mutations, which result in amino acid substitutions at positions 55, 57, or both. Different strains with single or double amino acid substitutions at these positions have been identified (Table 1). Based on homology to the *E. coli* DHPS, these point mutations appear to be in an active site of the enzyme involved in substrate binding; thus, amino

Table 1. Amino acid substitutions observed in *Pneumocystis jirovecii* [a]

DHPS genotype	Amino acid position 55	Amino acid position 57
Wild-type		
1	Threonine	Proline
Mutant		
2	Alanine	Proline
3	Threonine	Serine
4 (double mutant)	Alanine	Serine
Mixed		
5	Threonine	Proline/serine
6	Threonine/alanine	Proline/serine
7	Threonine/alanine	Proline
8	Alanine	Proline/serine

[a]DHPS, dihydropteroate synthase.

acid substitutions in these regions could result in structural changes that could interfere with substrate binding and enzyme activity (21). Likewise, similar point mutations in positions equivalent to this site in *Plasmodium falciparum* (15) and *Mycobacterium leprae* (28) confer sulfa resistance. Other mutations near this site also cause sulfa resistance in *S. pneumoniae* and *P. falciparum* (18).

However, the inability to reliably culture *Pneumocystis jirovecii* in a standardized in vitro culture system prevents the routine susceptibility testing of *Pneumocystis*. The lack of a standardized culture system also hampers research into the development and testing of new antimicrobial agents with anti-*Pneumocystis* activity, which highlights our reliance on TMP-SMX as the current mainstay of therapy. Thus, the clinical significance of these DHPS gene mutations must be inferred from correlating the clinical outcome with the presence of DHPS gene mutations in patients with PCP.

Association of Sulfamethoxazole and Dapsone with DHPS Gene Mutations

Several studies have consistently demonstrated a significant association between the use of TMP-SMX or dapsone for PCP prophylaxis in HIV-infected persons and the presence of DHPS gene mutations (Table 2) (19–25,29). One study extended these findings to the use of pyrimethamine plus sulfadoxine for PCP prophylaxis (30). Another study demonstrated an apparent reversal of the DHPS mutant-to-wild-type ratios after the use of TMP-SMX was restricted (31). In total, studies report >700 episodes of PCP, span a period from 1976 to 2001, and include patient data and clinical specimens from multiple cities in several different countries. Unfortunately, these studies used different criteria to define PCP prophylaxis with TMP-SMX or dapsone, which effectively limits attempts at data pooling for more direct and detailed analyses. In addition, most of the studies collected data by abstracting information from patient charts. Thus, these studies were unable to assess whether patients adhered to the prescribed prophylaxis. Nevertheless, seven of the nine studies found that most HIV-infected patients with a diagnosis of PCP who had been prescribed TMP-SMX or dapsone for PCP prophylaxis had *Pneumocystis* that contained DHPS mutations (range 19%–80%, Table 2) (19–24,30). Furthermore, eight of the nine studies reported that PCP patients for whom TMP-SMX or dapsone was prescribed were more likely to have *Pneumocystis* that contained DHPS mutations than were patients for whom these medications were not prescribed (19–25,30).

Of note, in all nine studies, DHPS mutations were observed in PCP patients who were not currently receiving TMP-SMX or dapsone. Whether these patients who failed to meet the defined criteria for TMP-SMX or dapsone prophylaxis had ever received one of these medications for prophylaxis or had received TMP-SMX for a reason other than PCP prophylaxis at some point during their lives was difficult to assess with any degree of confidence. Nevertheless, most of the studies found that only a minority of PCP patients who had not been prescribed TMP-SMX or dapsone for PCP prophylaxis had *Pneumocystis* that contained DHPS mutations. The study that reported the highest proportion (48%) used both chart abstraction and patient interview as sources of clinical information regarding PCP prophylaxis (23). This study also used a broad definition of PCP prophylaxis, including patient report of TMP-SMX use for prophylaxis at any time in life. Thus, despite rigorous attempts to document TMP-SMX or dapsone use for PCP prophylaxis and with the broadest definition of prophylaxis applied, nearly half of the patients without TMP-SMX or dapsone use had evidence of DHPS mutations on their clinical PCP specimen. Among the 26 patients with a new diagnosis of HIV infection at the time PCP was diagnosed and who thus had never received PCP prophylaxis, 14 (54%) had *Pneumocystis* that contained DHPS gene mutations. The specific city of residence was also an independent predictor associated with the risk for *Pneumocystis* that contained DHPS gene mutations. Patients who lived in San Francisco were five times more likely, and patients who lived in Seattle were more than three times as likely to have mutant DHPS than patients who resided in Atlanta, even when factors including sulfonamide or dapsone PCP prophylaxis and prior PCP were controlled for. The presence of DHPS mutations in patients without prior TMP-SMX or dapsone use for PCP prophylaxis, the absence of similar mutations in *Pneumocystis* isolated from other mammalian species, and the impact of geography on DHPS genotype have substantial implications for disease transmission (i.e., person-to-person transmission) that are beyond the scope of this review (32–35).

Table 2. Association between sulfonamide or sulfone for PCP prophylaxis and DHPS gene mutations[a]

Author (y) (ref)	PCP cases, no.	Location (time period), country	Prophylaxis[b] definition (source of information)	DHPS mutations among persons using prophylaxis N (%)	DHPS mutations among persons not using prophylaxis N (%)	p value
Kazanjian (1998) (19)	27 (20 HIV-infected)	Ann Arbor, MI (1991–1997), USA		5/7 (71)[c]	2/20 (10)[c]	0.0032
		Indianapolis, IN (1976–1997), USA	At least 1 out of 4 months preceding PCP (chart)	5/7 (71)[d]	2/13 (15)[d]	0.022
Helweg-Larsen (1999) (20)	152	Copenhagen (1989–1999), Denmark	Exposure[e] (chart)	18/29 (62)	13/123 (11)	< 0.0001
			Prophylaxis[f] (chart)	5/7 (71)[g]	15/125 (12)	0.01
Ma (1999) (21)	37 (26 HIV-infected)	Bethesda, MD (1985–1998), USA	Any (chart)	11/14 (79)	2/18 (11)	< 0.001
Kazanjian (2000) (22)	97	Denver, CO, Indianapolis, IN, Boston, MA, Detroit, MI (1991–1997), USA	At least 1 out of 4 months preceding PCP (chart)	28/37 (76)	14/60 (23)	< 0.001
Huang (2000) (23)	111	Atlanta, GA, Seattle, WA, San Francisco, CA (1996–1999), USA	Ever (interview), Any in the 3 months preceding PCP (chart and interview)	57/71 (80)	19/40 (48)	< 0.001
Visconti (2001) (24)	20	Rome (1992–1997), Italy	(Chart)	4/5 (80)	4/15 (27)	0.031
Ma (2002) (25)	107	Milan (1994–2001), Italy	Any in the 6 months preceding PCP (chart)	6/31 (19)	3/76 (4)	0.017
Costa (2003) (29)	89 (83 HIV-infected)	Lisbon (1994–2001), Portugal	Prophylaxis[h] Exposure[i]	6/17 (35)	18/72 (25)	0.39
Nahimana (2003) (30)	158 (120 HIV-infected)	Lyon (1993–1996), France	Prophylaxis[j]	16/20 (80)	41/138 (30)	< 0.001

[a]PCP, *Pneumocystis* pneumonia; dihydropteroate synthase (DPHS); TMP-SMX, trimethoprim-sulfamethoxazole.
[b]Prophylaxis refers to persons using TMP-SMX or dapsone who met the specific criteria described.
[c]The seven specimens with DHPS gene mutations were from 1995 to 1997.
[d]Analysis restricted to 20 HIV-infected persons.
[e]Exposure, continuous use for at least 1 week at any time after the diagnosis of HIV infection.
[f]Prophylaxis at least 8 weeks preceding PCP.
[g]Analysis restricted to patients receiving TMP-SMX or dapsone prophylaxis versus no prophylaxis.
[h]Prophylaxis, adherence to the same regimen for a minimum of 2 months preceding PCP.
[i]Exposure, use for at least 1 week in the 6 months preceding PCP.
[j]Prophylaxis 3 months preceding PCP. Includes patients who received pyrimethamine/sulfadoxine for PCP prophylaxis.

Lack of Association of Trimethoprim with DHFR Gene Mutations

Trimethoprim inhibits another of the integral enzymes in folate synthesis, DHFR (Figure). In other microorganisms, point mutations in the DHFR gene are an important mechanism of drug resistance. This finding has led researchers to examine the DHFR gene of *P. jirovecii* to evaluate whether DHFR mutations contribute to TMP-SMX resistance. To date, two studies have failed to demonstrate an association between the use of TMP-SMX for PCP prophylaxis in HIV-infected persons and the presence of DHFR gene mutations (21,36). In one study, 36 of 37 specimens (from 35 patients, 26 of whom were HIV-infected) demonstrated identical DHFR sequences, with a single specimen showing one synonymous nucleotide change (21). In the second study, 16 (59%) of 27 specimens (from 27 patients, 19 of whom were HIV-infected) had DHFR gene mutations, 14 had synonymous changes, and 2 had nonsynonymous changes (36). Neither of the two patients whose PCP specimen had nonsynonymous DHFR changes had prior exposure to DHFR inhibitors, yet both patients were treated successfully for PCP with TMP-SMX. In addition, this study aligned the *Pneumocystis* DHFR sequences with those of *E. coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Plasmodium falciparum* and reported that the observed nonsynonymous changes in *Pneumocystis* DHFR were not in the highly conserved regions of the enzyme as are the amino acid substitutions that confer resistance to TMP (or pyrimethamine) in these other organisms. Thus, the presence and association of DHPS, but not DHFR, gene mutations with the use of specific PCP prophylaxis regimens argue strongly both for the importance of SMX and dapsone against *Pneumocystis* and the central role of DHPS mutations in the potential development of TMP-SMX or dapsone resistance.

Clinical Importance of DHPS Gene Mutations in *Pneumocystis jirovecii*

A number of studies have examined the effect of DHPS gene mutations on clinical outcomes such as death, death specifically attributable to PCP, and PCP treatment failure with TMP-SMX or dapsone plus trimethoprim (Table 3) (19–22,24,25,37–39). Whether the presence of DHPS gene mutations confers clinical resistance to TMP-SMX or dapsone plus trimethoprim for PCP treatment remains unclear and requires further study. In a multivariate analysis, Helweg-Larsen and colleagues found that DHPS mutations were an independent predictor associated with increased death rates (20). In this study, DHPS mutation was the strongest predictor of death, and patients who had *Pneumocystis* that contained DHPS mutations had a greater than threefold increased risk for death within 3 months compared to patients with the wild-type DHPS, after important mortality cofactors such as age, $CD4^+$/cell count, and arterial oxygen partial pressure (PaO_2) were controlled for. Whether this increased death rate was due to failure of TMP-SMX for PCP treatment is unclear. In fact, 12 (63%) of 19 PCP patients with *Pneumocystis* that contained DHPS gene mutations responded to PCP treatment with TMP-SMX. Kazanjian and co-workers found that the presence of DHPS mutations was associated with an increased risk for PCP treatment failure with TMP-SMX or dapsone plus trimethoprim (22). In univariate analysis, PCP patients who had *Pneumocystis* that contained DHPS gene mutations had a greater than twofold increased risk for treatment failure with one of these regimens, compared to patients with the wild-type DHPS. In this study, treatment failure was defined as worsening of clinical features after 7 days of therapy, failure to improve after 10 days of therapy, or a change in therapy because the treating physician perceives failure. Patients who responded clinically to therapy but who switched therapies because of adverse effects were considered to have been treated successfully. Similar to the findings of Helweg-Larsen, most patients

Table 3. Association between DHPS gene mutations and important clinical outcomes[a]

Author (y) (ref)	PCP cases, no.	DHPS mutations, no.	Increased death rate?	Increased PCP treatment failure?	Comments
Kazanjian (1998) (19)	27	7	NA	NA	Both patients with DHPS mutations who were treated with TMP-SMX responded to treatment.
Mei (1998) (37)	2	2	NA	NA	2 patients with DHPS mutations were treated with TMP-SMX: 1 did not respond to TMP-SMX (but responded to pentamidine); 1 responded to TMP-SMX.
Helweg-Larsen (1999) (20)	144	29	Yes[b] 3 months	NA	DHPS mutation was an independent predictor associated with increased deaths (OR = 3.1, p = 0.01). 19 patients with DHPS mutations were treated with TMP-SMX: 7 died; 12 (63%) responded and survived.
Ma (1999) (21)	37	13	No	NA	
Kazanjian (2000) (22)	97	42	No[c] 4 weeks	Yes[d]	Patients with DHPS mutations were more likely (RR = 2.1, p = 0.01) to fail TMP-SMX or dapsone-containing treatment. Nevertheless, 15 (71%) of 21 patients with DHPS mutations who were treated with TMP-SMX or dapsone-containing regimen responded to treatment.
Takahashi (2000) (38)	22	4	NA	Yes	All 4 patients with DHPS mutations who were treated with TMP-SMX did not respond to treatment.
Navin (2001) (39)	136	97	No[e] weeks	No[f]	66 patients with DHPS mutations were treated with TMP-SMX: 56 (85%) responded.
Visconti (2001) (24)	20	8	NA	No	1 of 3 patients with DHPS mutations did not respond to TMP-SMX treatment.
Ma (2002) (25)	107	9	No[g] 4 weeks	No	

[a]DHPS, dihydropteroate synthase; PCP, *Pneumocystis* pneumonia; TMP-SMX, trimethoprim-sulfamethoxazole; NA, not available.
[b]Assessed at 3 months.
[c]Assessed at 4 weeks.
[d]Defined as the following: a) deterioration after 7 days of therapy (worsening clinical features or gas exchange parameters—alveolar-arterial O_2 gradient increase ≥20 mm Hg from baseline—when available); b) failure of clinical findings to improve after 10 days of therapy; c) physician perception of failure.
[e]Assessed at 6 weeks. Results were similar whether deaths were defined as from all cause or restricted to cases in which PCP was the primary cause of death.
[f]PCP treatment response defined as the following: a) patient completed full course of initial treatment and responded; b) patient responded sufficiently to be discharged on oral medication; c) patient responded to initial treatment but was given another medication because of adverse effects. Results were similar when analysis was restricted to patients who had received at least 7 days of initial PCP treatment.
[g]Assessed at 4 weeks. Deaths included were restricted to cases in which PCP was the primary cause of death.

with *Pneumocystis* that contained DHPS gene mutations responded to PCP treatment with TMP-SMX or dapsone plus trimethoprim. Overall, 15 (71%) of 21 PCP patients with *Pneumocystis* that contained DHPS gene mutations responded to PCP treatment with one of these two regimens. In addition, this study found no association between the presence of DHPS mutations and death at 4 weeks. In contrast to these prior two studies, Navin and colleagues found no association between the presence of DHPS mutations and overall number of deaths at 6 weeks, death attributable specifically to PCP, or PCP treatment failure (39). Overall, 16 (17%) of 94 PCP patients with DHPS mutations died compared to 9 (25%) of 36 PCP patients with wild-type DHPS ($p = 0.30$). Similarly, seven patients (7%) with PCP with DHPS mutations died as a result of PCP compared to four patients (11%) with wild-type DHPS. Among the 66 patients with PCP with DHPS mutations who were treated with TMP-SMX, 56 (85%) responded to this treatment. In this study, patients were classified as having been successfully treated if they completed a full course of therapy and responded or if they responded sufficiently to be switched from intravenous to oral therapy and be discharged. Similar to the Kazanjian study, patients who responded clinically to therapy but who switched therapies because of adverse effects were considered to have been treated successfully. This noted TMP-SMX response rate was significantly better than the rate for patients with DHPS mutation who were treated with intravenous pentamidine or clindamycin plus primaquine (14 [50%] of 28) and for patients with the wild-type DHPS who were treated with TMP-SMX (23 [64%] of 36). These results were similar when the analysis was restricted to patients who had been treated for at least 7 days with their initial therapy. Although these three patient groups did not differ in terms of age, CD4-cell count, serum albumin, serum lactate dehydrogenase (LDH), or proportion who required corticosteroids, no multivariate analysis was performed to determine independent predictors associated with death (or PCP treatment failure). Instead, a series of stratified analyses were performed and failed to detect any subsets of PCP patients in whom DHPS mutations were associated with a worse outcome.

Summary and Future Directions

Whether *Pneumocystis* DHPS gene mutations confer clinical resistance to TMP-SMX or dapsone plus trimethoprim for PCP treatment remains unclear. Published studies offer conflicting results. Each study used different definitions for PCP prophylaxis and PCP treatment success or failure, and each examined patient deaths at different timepoints, with different methods of statistical analysis. These methodologic differences limit attempts at data pooling for more direct and detailed analyses. The outcome of HIV-infected patients with PCP is a complex issue, with multiple factors affecting death, including those related to the patient (e.g., age), the patient's overall health status (e.g., serum albumin), the underlying HIV/AIDS (e.g., coexisting opportunistic infections or conditions), and, of course, those specific factors related to PCP (e.g., disease severity, presence of respiratory failure, need for mechanical ventilation, and development of serious complications such as pneumothorax). In each individual report, the overall number of patients studied and the subset of patients who had *Pneumocystis* that contained DHPS mutations and were treated with TMP-SMX or dapsone plus trimethoprim were too small to account for these factors and to detect small differences in outcome that may be related to drug resistance. Furthermore, these and future observational studies that examine DHPS genotype and PCP treatment outcome are complicated by the absence of validated PCP clinical treatment guidelines, practice standards, and definitions of treatment success or failure.

While the declining incidence of PCP in the United States and Europe, as a result of combinations of antiretroviral therapy and PCP prophylaxis, might lessen the enthusiasm for continued study of this issue, brief consideration of a number of factors that warn of a future "perfect storm" suggests that continued study is important. First, most HIV-infected persons worldwide reside in sub-Saharan Africa, Southeast Asia, and Latin America, places where access to antiretroviral therapy and PCP prophylaxis are limited. Second, PCP is increasingly being recognized as an important cause of illness in these regions. In many of these regions, programs to use TMP-SMX as multiopportunistic infection prophylaxis are being implemented, and *Pneumocystis* that contains DHPS mutations can be expected. Next, the treatment options for PCP in these regions are often limited to TMP-SMX, since regimens such as pentamidine, clindamycin plus primaquine, trimetrexate, and atovaquone are unavailable. The existence of TMP-SMX–resistant *Pneumocystis* in these regions, combined with the general absence of invasive diagnostic procedures (e.g., bronchoscopy that might establish an earlier diagnosis of PCP when the outcome is better) and intensive care facilities (e.g., mechanical ventilation that might support patients until PCP treatment can be effective), stresses the importance of further study (40).

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Frank D. Duncan, Jr. (b. 1916), Sisters of Mercy (1944).
Watercolor on paper, 39.37 cm x 49.53 cm. U.S. Army Center of Military History, Washington, DC, USA

Strain Typing Methods and Molecular Epidemiology of *Pneumocystis* Pneumonia

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Pneumocystis pneumonia (PCP) caused by the opportunistic fungal agent *Pneumocystis jirovecii* (formerly *P. carinii*) continues to cause illness and death in HIV-infected patients. In the absence of a culture system to isolate and maintain live organisms, efforts to type and characterize the organism have relied on polymerase chain reaction–based approaches. Studies using these methods have improved understanding of PCP epidemiology, shedding light on sources of infection, transmission patterns, and potential emergence of antimicrobial resistance. One concern, however, is the lack of guidance regarding the appropriateness of different methods and standardization of these methods, which would facilitate comparing results reported by different laboratories.

Pneumocystis pneumonia (PCP) has been known for many years to be a disease of immunocompromised persons. Before the AIDS epidemic, it had been reported as a cause of death in malnourished infants (1). No standardized in vitro propagation system is currently available; consequently, much of the basic biology and epidemiology of *Pneumocystis* spp. remains poorly understood. Advances made over the last 15 years have been largely due to the use of molecular biologic approaches.

For almost 80 years, *Pneumocystis jirovecii* (formerly *carinii*) was considered to be a protozoan. In 1988, DNA studies clearly demonstrated that it was not a single species but a complex group of eukaryotic microorganisms, which were assigned to the kingdom Fungi (2–4) at the branch point between *Ascomycota* and *Basidiomycota* (5).

Many genetic typing methods use DNA sequencing approaches, but others use specific gene probes, single-strand conformation polymorphism (SSCP), or restriction fragment length polymorphisms (RFLP). Genetic typing has shown *Pneumocystis* biodiversity (6), environmental reservoirs (7,8), person-to-person transmission (9,10), recurrent infections (11), subclinical colonization and carriage (12–14), clinical manifestations (15), and sulfa exposure and suspected treatment or prophylaxis failures (16–19). These studies changed our epidemiologic understanding of PCP, and more studies now suggest collectively that a number of clinical PCP cases are newly acquired rather than activated latent infections (9–12,18).

In laboratories around the world, a number of typing procedures, each with its own strengths and weaknesses, are in use to address the clinical and epidemiologic issues discussed above. In the following sections, we discuss the most common methods, along with examples of how they have been used in molecular epidemiologic studies of PCP.

Different Typing Methods

A variety of typing methods have been used for *Pneumocystis* genetic analysis, and a large number of gene loci have been examined. We focus on the methods and genes that have been most widely used for molecular epidemiologic analyses or have the greatest potential application.

DNA Sequence Analysis

Direct DNA sequence analysis is the most common approach currently used for *Pneumocystis* biodiversity and molecular typing studies. Sequence analysis of the thymidylate synthase (TS) and superoxide dismutase (SODA) gene loci, the EPSP synthase domain of the multi-

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functional arom gene, and the mitochondrial small subunit ribosomal RNA (mt SSU rRNA) locus have been used to distinguish *Pneumocystis* species from diverse mammalian hosts (6,20,21). Because of the generally low sequence divergence among *P. jirovecii* isolates at these loci, they are not highly discriminative for *P. jirovecii* typing. Several additional loci, however, have proved useful for molecular epidemiologic applications (7–19). These include the internal transcribed spacer (ITS) regions of the nuclear rRNA operon (9,10,15), the mitochondrial large subunit ribosomal RNA locus (mt LSU rRNA) (7,8,10), and the dihydropteroate synthase (DHPS) gene (10,16–19).

ITS1 and ITS2

The ITS1 sequence is located on the nuclear rRNA operon between the genes of the 18S rRNA and the 5.8S rRNA, and ITS2 is located between the genes of the 5.8S rRNA and the 26S rRNA (22). These noncoding loci are spliced during rRNA synthesis. They show a high level of polymorphism, which has been used for genetic typing applications. The first ITS typing system was developed by Lu et al. (22). Using their nomenclature, in which ITS1 alleles are designated with an uppercase letter and ITS2 alleles with a lowercase letter, 15 ITS1 alleles (from A to O) and 14 ITS2 alleles (from a to n) have been described. Based of this amount of DNA polymorphism, a total of 210 *P. jirovecii* types are theoretically possible, with 59 types reported by these authors (22,23).

A second ITS typing scheme was developed by Tsolaki et al. (24–26) and is based on nucleotide variation at four positions in the ITS1 and at six positions in the ITS2. According to their nomenclature scheme, ITS1 alleles are designated by using an uppercase letter associated with a numerical subscript, and ITS2 alleles are designated by using a lowercase letter also associated with a numerical subscript. These researchers described six ITS1 alleles and nine ITS2 alleles; these numbers allow for up to 54 potential *P. jirovecii* ITS types, should all possible combinations exist. Laboratories currently using this typing scheme have reported ≈40 different *P. jirovecii* ITS types. The most frequent types are B_1a_3 and B_2a_1, which have both been identified in one third of all *P. jirovecii* isolates typed to date.

More recently, Nimri et al. (27) added to the count of Lee et al. by identifying 12 previously unreported ITS1 alleles and 16 previously unreported ITS2 alleles. In the study by Nimri et al., 36 ITS types were noted in 180 sequences examined from 60 samples. Although the typing methods of Lee et al. and Tsolaki et al. are not strictly identical, a general correspondence between *P. jirovecii* ITS types can be observed with either method. To date, approximately 87 unique ITS types have been identified by the two methods.

mt LSU rRNA

The amount of polymorphism reported at this locus is substantially less than that reported for ITS; nevertheless, the variation observed has helped address a number of important epidemiologic questions. The original PCR assay developed for this locus was a single-round PCR that generated a fragment of ≈360 bp (28). A nested PCR assay has also been developed (25), which has an increased sensitivity and specificity. Recently, this test was used to distinguish subclinical carriage from clinical disease (14).

Polymorphism at this locus is routinely reported at two nucleotide positions (85 and 248), showing six unique genotypes. A third variable position has been reported but is rarely seen. Mitochondrial DNA has long been accepted and used as a practical and reproducible tool to evaluate intraspecific variation. Since multiple mitochondria are present in individual organisms but display the same haplotype, mitochondrial loci are more easily detected by PCR than single-copy nuclear genes, which results in generally higher PCR sensitivities.

The DHPS Locus

The DHPS locus encodes the key enzyme that is targeted by sulfonamide antimicrobial drugs. Consequently, typing efforts involving this gene have been directed primarily at demonstrating an association between treatment or prophylaxis failures and the specific mutations observed at this locus (16–19). Polymorphism at this locus has been observed primarily at amino acid positions 55 and 57, where nonsilent mutations have been shown to correlate with sulfonamide exposure and prophylaxis and treatment failure. A nested-PCR assay has been developed for this locus (29), with modifications suggested by other investigators (18). This assay is highly sensitive and specific for *P. jirovecii* in patients with clinical PCP. In addition to the position 55/57 mutations, nucleotide polymorphism has also been observed at several other sites in the gene (30). These mutations have not been shown to result in amino acid substitutions and have not been correlated with adverse clinical outcomes.

Multitarget PCR-SSCP

A second method for molecular typing *P. jirovecii* involves single-strand confirmation polymorphism (SSCP) analysis (31,32). The method consists of PCR amplification of four variable regions of *P. jirovecii* (Table), then detecting polymorphism by observing migration pattern variation in gel electrophoresis. The variable regions analyzed include ITS1, the intron of the nuclear 26S rRNA gene (26S), the variable region of the mt LSU rRNA, and the region surrounding an intron of the β-tubulin gene (β-tub).

A variable region amplified from a clinical specimen of a given patient with PCP can generate a simple or complex

Table. Primer sets for polymerase chain reaction amplification of *Pneumocystis* gene loci commonly used for molecular typing[a]

Gene locus		Primer sequence	Reference
β-tubulin			
Forward		5′ TCA TTA GGT GGT GGA ACG GG 3′	(31)
Reverse		5′ ATC ACC ATA TCC TGG ATC CG 3′	(31)
DHPS (nested)			
1st round	DHPS F1	5′ CCT GGT ATT AAA CCA GTT TTG CC 3′	(28)
	DHPS B_{45}	5′ CAA TTT AAT AAA TTT CTT TCC AAA TAG CAT C 3′	(29)
2nd round	DHPS A_{HUM}	5′ GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C 3′	(29)
	DHPS BN	5′ GGA ACT TTC AAC TTG GCA ACC AC 3′	(29)
ITS (nested)			
1st round	1724F	5′ AAG TTG ATC AAA TTT GGT C 3′	(22)
	ITS2R	5′ CTC GGA CGA GGA TCC TCG CC 3′	(22)
2nd round	ITS1F	5′ CGT AGG TGA ACC TGC GGA AAG GAT C 3′	(22)
	ITS2R1	5′ GTT CAG CGG GTG ATC CTG CCT G 3′	(22)
ITS (nested)			
1st round	N18SF	5′GGT CTT CGG ACT GGC AGC 3′	(26)
	N26SRX	5′ TTA CTA AGG GAA TCC TTG TTA 3′	(26)
2nd round	ITSF3	5′ CTG CGG AAG GAT CAT TAG AAA 3′	(24)
	ITS2R3	5′ GAT TTG AGA TTA AAA TTC TTG 3′	(24)
MSG			
Forward	GK242	5′ TAT TTC TTG TAT CTA TGC GCT 3′	(33)
Reverse	GK244	5′ TCC GCG CAA AAA TAA GCA CT 3′	(33)
mt LSU rRNA (nested)			
1st Round	pAZ102-H	5′ GTG TAC GTT GCA AAG TAC TC 3′	(28)
	pAZ102-E	5′ GAT GGC TGT TTC CAA GCC CA 3′	(28)
2nd Round	pAZ102-X	5′ GTG AAA TAC AAA TCG GAC TAG G 3′	(25)
	pAZ102-Y	5′ TCA CTT AAT ATT AAT TGG GGA GC 3′	(25)
Nuclear 26S rRNA			
Forward		5′ GAA GAA ATT CAA CCA AGC 3′	(31)
Reverse		5′ ATT TGG CTA CCT TAA GAG 3′	(31)

[a]DHPS, dihydropteroate synthase; ITS, internal transcribed spacer; MSG, major surface glycoprotein; mt LSU, mitochondrial large subunit.

SSCP pattern. Simple patterns are made of two bands and correspond to a single allele of the genomic region. Complex patterns are made of more than two bands and have been shown to correspond to the superimposition of two simple patterns and the presence of two or, rarely, three alleles of the region (32).

According to their SSCP results, different categories of specimens can be distinguished (32). A specimen harboring a single allele at each of the four genomic regions is presumably infected with a single *P. jirovecii* type, and each combination of four simple SSCP patterns defines a type (Figure 1). Studies strongly suggest that a patient harboring two or more alleles of at least one of the genomic regions is coinfected with several *P. jirovecii* types (31). Analysis of the alleles and their abundance within the complex patterns allows identification of the coinfecting types in ≈60% of specimens coinfected with two types. Specimens producing at least one complex SSCP pattern made of three simple patterns are presumably infected with at least three types, which cannot be identified. However, the SSCP results of the latter specimens are also informative, as they often allow exclusion of certain specific types. Among 430 specimens from 15 hospitals in five European countries, three to five different simple SSCP patterns could be identified for each genomic region and 43 different *P. jirovecii* types. Thirty percent of the patients were infected with a single *P. jirovecii*, 45% with two types, and 25% with three (31,32).

Major Surface Glycoprotein Expression Site Typing

A third, recently reported typing method relies on identifying the number of tandem repeats in the intron of the expression site of the major surface glycoprotein (MSG) of *P. jirovecii* (Table) (33). Unlike the other currently available typing methods, which rely on identifying single nucleotide polymorphisms or combinations of such polymorphisms, this method relies on characterizing the size of a region of this intron. Within this region, different *P. jirovecii* isolates have two to six copies of a 10-nt sequence. Typing can be performed by amplifying this region with PCR using primers flanking this region and running the PCR product on a high-resolution acrylamide gel that can separate fragments that differ in size by a few base pairs (33).

PCR followed by electrophoresis can be used to rapidly determine the number of repeats present in the intron.

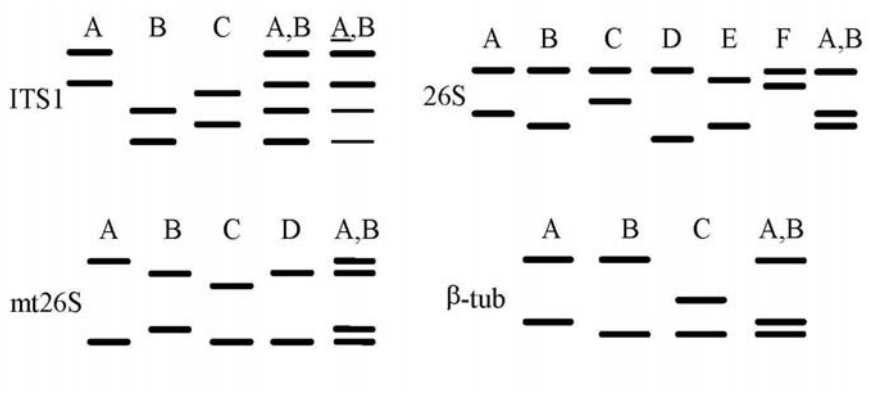

Figure 1. Schematic representation of the single-strand conformation polymorphism (SSCP) patterns of four variable regions used to type *Pneumocystis jirovecii*. Each lane corresponds to a hypothetical sample. All simple patterns with two bands for each region are shown. Each uppercase letter represents a simple SSCP pattern. For each region, the complex SSCP pattern A,B corresponding to the superimposition of simple patterns A and B is represented. The complex ITS1 pattern A,B, is demonstrated, in which pattern A is more abundant than pattern B. Reprinted with permission from reference 32. Hauser et al. 2001, AIDS:15(4):461–466.

Because the expression site of the MSG (unlike the MSG itself) is present as a single copy per organism (34,35), a given strain of *P. jirovecii* will have a unique number of repeats per organism. Infections with more than one strain of *P. jirovecii*, which occur frequently in HIV-infected patients (20%–70% of patients) can be easily detected with this method if the different strains have a different number of repeats, since PCR amplification will result in multiple bands corresponding to the different sizes of the repeats (Figure 2).

The utility of this typing method can be enhanced by sequencing the amplified PCR product because the 10-bp repeats can have one of three sequences (types 1, 2, and 3), which differ from each other by a single nucleotide. Isolates with the same number of repeats can potentially be distinguished from each other by different patterns of repeat types (e.g., three repeats of type 1, 1, 2 are different from three repeats of type 1, 2, 2). However, in isolates with multiple strains of *P. jirovecii*, as determined by quantifying the number of repeats, directly sequencing the PCR product will sequence only the predominant strain. Because adding or deleting 10 bases will shift the homologous sequences by 10, bases will be out of alignment downstream of the shift, making sequencing difficult. Subcloning, followed by sequencing, must be used in these circumstances to determine sequences of minority strains.

Analyzing the sequence of repeats also may provide insight into the evolution of *P. jirovecii*. The single base-pair changes seen in repeats likely occurred on a single occasion: since such a mutation appears to be rare (only two unique mutations have been identified) and likely does not confer a biologic advantage to the organism, the same mutation would not likely occur in the same location at different times. Thus, organisms with the 1, 1, 2 pattern of three repeats must have derived from a parental strain with a 1, 2 pattern of two repeats, rather than a 1, 1 pattern of two repeats, both of which have been seen in separate isolates (Figure 3). An analysis of multiple isolates from around the world will potentially provide information about the evolution and spread of *P. jirovecii*. If archival isolates can be identified and examined, we may gain additional information about the evolution of the organism over time.

Because this method was only recently described, it has been used by one group to date. In these limited studies of 147 samples from the United States and Europe, three repeats were most commonly seen, either alone or in combination (33). Two and four repeats were seen less frequently, five repeats were seen only in two mixed infections, and six repeats were seen in two isolates, one of which was in a mixed infection with three and four repeats. No pattern related to time of obtaining the sample (1974–2001) or geographic location was identified. Coinfection with more than one strain was identified in 43% of the 147 samples with this method. Additional experience with this typing method, alone and in combination with other methods, is needed to better evaluate its utility in understanding the importance of strain variation and in studying the epidemiology and biologic variability of this organism.

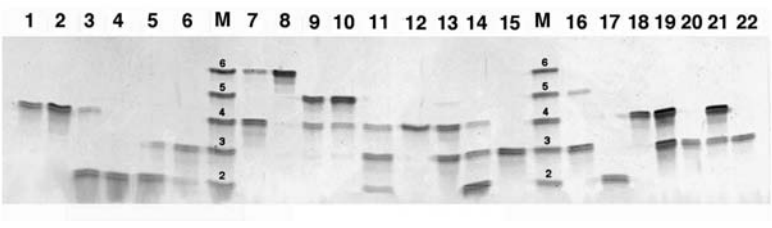

Figure 2. Representative denaturing gel electrophoresis analysis of *Pneumocystis jirovecii* tandem repeats in clinical isolates. Numbers above each lane represent individual isolates. Lane M is a mixture of polymerase chain reaction products from five isolates, of which the number of repeats was 2, 3, 4, 5, and 6 (shown above DNA bands), as determined by sequencing. Reprinted from (33) with permission from the University of Chicago Press.

Assay Stability and Reproducibility

A potential limitation in typing that should be considered in evaluating the various approaches is the shortage of information available on marker stability and assay stability. While these data are generally lacking for most of the commonly used approaches, efforts have been made to evaluate and validate stability over time of multitarget PCR-SSCP (32). Specifically, pairs of specimens collected from the same patients during a single PCP episode were analyzed to evaluate the stability of genetic markers. Markers remained stable throughout the 8-week study, which suggests that this method was valid for most clinical applications. Less formal evaluations with direct DNA sequencing show a similar level of stability with the conserved gene loci mt LSU rRNA and DHPS (C.B. Beard, unpub. data). Unexpectedly high rates of ITS variability have been reported in samples collected from the same patient at different times during a single disease episode (36). Several possible explanations were proposed, including quantitative changes in the relative abundance of mixed *P. jirovecii* populations, sampling bias, intrinsic instability of the gene locus, and methodologic artifact. Others have observed similar variation patterns at this locus (C.B. Beard, unpub. data), and the explanation is a subject of debate.

Typing-related Analyses

Two other molecular approaches may address clinical or epidemiologic questions. These applications differ from most typing efforts in that the usual purpose of typing is to evaluate genetic polymorphism, whereas these assays examine organism numbers and viability. A recently developed quantitative PCR assay based on a conserved region of the MSG gene provides a sensitive method for quantifying organism load in oral washes of patients with suspected PCP (37,38). In the absence of a reliable culture system, PCR-based viability assays directed against mRNA targets have been also been developed (39,40). Since these applications go beyond the scope of this article, they will not be discussed further; however, they are useful for clinical and molecular epidemiologic studies.

Best Typing Method

Much consideration has been given to the question of the best genes and best approaches for molecular typing. The answer in most cases will be determined by the typing objective. One consideration is the evolutionary rate of the gene. In most eukaryotic organisms, mitochondrial DNA has been reliable for examining intraspecific variation. In *P. jirovecii*, the mt LSU rRNA locus has generated useful data for addressing specific epidemiologic questions (7,8,10). A greater level of intraspecific variation (22–27), however, can be detected by using the ITS locus because of its more rapid evolution. Two potential complications associated with the ITS locus are related to assay stability: the specific gene sequence, which includes a polynucleotide stretch of ≈9–12 thymidines that can lead to *Taq* polymerase error during amplification, and the possibility that multiple genotypes occurring in a single isolate could result from two indistinguishable sources, coinfecting *P. jirovecii* strains, or diploid heterozygote organisms in the sample. These concerns do not imply that the locus should not be used but only that these possibilities should be considered when interpreting the data.

The mt LSU rRNA and ITS loci are frequently used because they are not assumed to be under genetic selection and are therefore useful for elucidating molecular evolutionary phenomena that provide the basis for understanding the history of circulating strains. Sometimes, however, typing is employed specifically to determine the existence of genetic selection, such as that induced by exposure to antimicrobial agents (16–19). Care must be taken in drawing inferences from differences observed at loci that are under genetic selection, since selection can confound inferences concerning strain differences.

DNA sequencing provides the most exhaustive amount of information about any particular DNA fragment, but it is expensive and labor-intensive. Fragment analysis

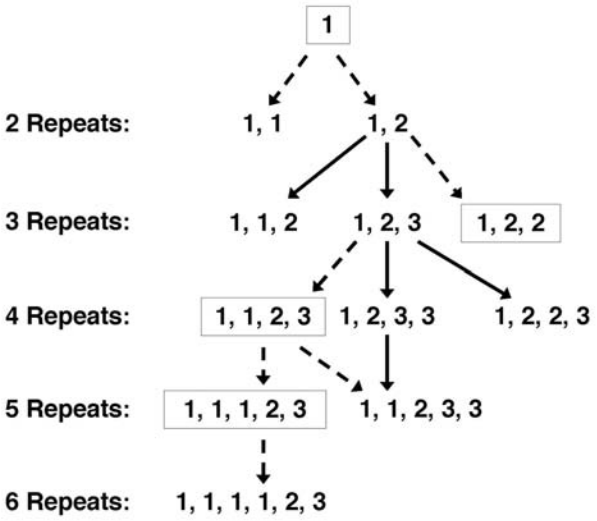

Figure 3. A model for the evolution of tandem repeats in *Pneumocystis jirovecii*, for repeat patterns that have been identified to date. The number of repeats is indicated on the left. The specific pattern of repeats is indicated on the right. The numbers 1, 2, and 3 represent three different repeat types with sequence variation in the first and fourth nucleotides. The repeat patterns that were not identified in this study but are postulated to exist, on the basis of identified patterns, are boxed. Solid arrows indicate potential evolution between isolates that have been identified, and dashed arrows indicate evolution between postulated isolates. Reprinted from (33) with permission from the University of Chicago Press.

methods such as SSCP are simpler and less expensive, but they rely on having sequence data to characterize the patterns observed. SSCP is also limited in its ability to interpret new genotypes. Both multilocus DNA sequencing and multitarget PCR-SSCP can incorporate information from multiple genetic loci, which allows higher discriminating power to identify strain differences. Low-resolution methods (e.g., RFLP) are best used when the goal is to look only for specific RFLP-defined mutations. Either SSCP or DNA sequencing is suitable for most molecular epidemiologic studies.

Need To Standardize Reporting

The greatest need in standardization is adopting well-defined sequence types. A good example is the convention used with discussing DHPS mutations, in which the nucleotide or amino acid position is given, along with the specific identity (e.g., Thr > Ala at position 55 and Pro > Ser at position 57 to denote the DHPS double mutant genotype). Using arbitrarily defined numbers or other alphanumeric characters to define genotypes should be avoided, except perhaps for brevity in an article in which observed genotypes are all defined by nucleotide position and identity in a table. The nomenclature systems developed for the ITS locus should be reevaluated; authors' intentions were good, but they did not account for the variation possible at that locus. A better nomenclature scheme would be to use the specific nucleotide position or variant relative to the original GenBank consensus sequence for that locus, as is typically done when reporting mutations. With the *Pneumocystis* genome project under way and as more genes are cataloged, nomenclature will need to be standardized further, such as with three-letter designations for genes. Within the *Pneumocystis* field, current practice is to use four or more letters to define some loci (e.g., DHFR, DHPS, MTLSU rRNA). Nomenclature standardization, however, should not affect the adoption of standardized typing methods using selected gene targets for specific molecular epidemiologic applications.

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West Nile Virus Economic Impact, Louisiana, 2002

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West Nile virus (WNV) is transmitted by mosquitoes and can cause illness in humans ranging from mild fever to encephalitis. In 2002, a total of 4,156 WNV cases were reported in the United States; 329 were in Louisiana. To estimate the economic impact of the 2002 WNV epidemic in Louisiana, we collected data from hospitals, a patient questionnaire, and public offices. Hospital charges were converted to economic costs by using Medicare cost-to-charge ratios. The estimated cost of the Louisiana epidemic was $20.1 million from June 2002 to February 2003, including a $10.9 million cost of illness ($4.4 million medical and $6.5 million nonmedical costs) and a $9.2 million cost of public health response. These data indicate a substantial short-term cost of the WNV disease epidemic in Louisiana.

West Nile virus (WNV) is transmitted by mosquitoes and can cause illnesses ranging from simple fevers to encephalitis (1). The presence of this virus in the Western Hemisphere was first recognized in New York City in 1999 (2). In 2002, an epidemic of WNV illness focused in the midwestern United States resulted in 4,156 reported cases; 2,942 cases had central nervous system (CNS) illness (meningitis, encephalitis, or acute flaccid paralysis), and 284 died (3). A total of 329 persons with WNV disease were reported in Louisiana, with illness onsets from June to November. Among these, 204 had illnesses involving the CNS; 24 died (Louisiana Office of Public Health, unpub. data).

Economic data about epidemics are essential for estimating the costs and benefits of strengthening and maintaining prevention and control programs, improving existing surveillance systems, and introducing other proposed interventions, such as vaccines. Although some estimates exist of the economic impact imposed by diseases transmitted by mosquitoes (4–9), to our knowledge, no previous studies have assessed the costs of a WNV disease epidemic. We estimated the magnitude of the short-term economic costs of the 2002 WNV epidemic in Louisiana.

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Economic Model, Data, and Methods

We calculated the costs of the WNV epidemic as the sum of 1) medical costs (inpatient and outpatient); 2) nonmedical costs, such as productivity losses caused by illness and premature death, costs of transportation for a patient to visit a healthcare provider, and childcare expenses; and 3) costs incurred by public health and other government agencies for epidemic control. Data were gathered from hospitals in Louisiana that had WNV patients; a phone survey of WNV patients (all adult patients with nonfatal cases for whom phone numbers were available from the Louisiana Office of Public Health were included in the survey); and public offices, including the Louisiana Office of Public Health, state and local governments, and the Louisiana Office of Emergency Preparedness. Because information could not be gathered for all hospitalized patients and the patient questionnaire could not be administered to all reported patients, we extrapolated cost data, assuming that the costs for those with information were representative of those without information (the extrapolation method is described in Appendix 1).

We took a societal perspective, evaluating all costs regardless of who bore them. The costs were estimated from June 2002, when the epidemic was first recognized, until the last date we administered the phone survey, February 27, 2003, some 3 months after the onset of illness of the last reported patient. Intangible costs, attributable to factors such as pain and suffering, were not included.

Medical Costs

Inpatient Costs for Acute Care and Rehabilitation

In fall 2002 we requested information from Louisiana hospitals on the length of hospital stay and inpatient and outpatient treatment charges, including therapies at inpatient rehabilitation facilities for patients who met the case

definition of probable or confirmed WNV illness (http://www.cdc.gov/epo/dphsi/casedef/encephalitiscurrent.htm). To ensure patient anonymity, patient information from hospitals was given to the study investigators unlinked to personal identifiers, and only the 16 hospitals with more than three adult patients (>18 years old) were queried. Adults constituted 94% of reported WNV case-patients in Louisiana.

Twelve hospitals submitted information from 159 patients, including inpatient treatment charges for 119 patients and hospital outpatient treatment charges for 50 patients. Ten of these 50 patients had both inpatient and outpatient treatment charges. Patient charges included 65 inpatient treatment or service types, which we grouped for the analysis into eight categories (Table 1). For example, we pooled hospital charges originally listed as "pharmacy," "drugs," "injection," "medical/surgical supplies," "IV solutions," "IV therapy," and "prosthetic devices" into the category "pharmacy/medical supplies."

Because charges for healthcare products or services may not represent their true economic cost (Appendix 2), i.e., the opportunity cost of a resource used for producing goods, services, or both (10,11), we converted hospital charges to economic costs by using Medicare cost-to-charge conversion rates (12). Charges made by healthcare providers are generally higher than the cost of resources used (Appendix 2). For Louisiana, the cost-to-charge ratios were 0.410 for urban areas and 0.488 for rural areas (e.g., in urban areas, a $1 charge has an estimated $0.41 economic cost). Two of 12 participating hospitals were in rural areas.

Of 119 patients for whom inpatient treatment charges were available, 7 incurred costs for inpatient rehabilitation treatment. These inpatient rehabilitation treatment charges were provided by acute-care hospital–based rehabilitation centers. Charges were converted into costs as described above. The costs of treatment for the seven patients were then extrapolated to estimate the total costs for all CNS patients requiring rehabilitation by using the methods described earlier.

Outpatient Costs, Medication, and Durable Medical Equipment

Information for estimating medical doctor visit costs, outpatient rehabilitation treatment costs, and nonmedical costs, including productivity losses, was gathered by interviews using a questionnaire administered by telephone from December 7, 2002, until February 27, 2003 (questionnaire provided in Online Appendix [www.cdc.gov/ncidod/EID/vol10no10/03-0925_app.htm]). Phone numbers for 236 adult patients with nonfatal WNV cases were available from the Louisiana Office of Public Health. Of these 236 persons, 139 were interviewed, 46 did not answer the phone (at least three calls were made at different times of day), 4 were deceased, 2 denied WNV illness, and 16 refused to participate. Twenty-nine of the phone numbers were listed incorrectly or were disconnected.

We collected information about general practice, specialist, and outpatient rehabilitation treatment visits through the patient questionnaire. We estimated the costs for these visits by using a private health insurance claims database (Marketscan database 1999, The MEDSTAT Group, Inc., Ann Arbor, MI). This database is compiled from health insurance claims submitted to 40 self-insured employers and represents over 5 million covered lives across the United States. Average payments made to healthcare providers in the United States in 1999 were calculated for each service. Costs of specialist visits were estimated on the basis of relative prices compared to the national average payments for general practitioners. Relative prices for medical specialists and hospital-based specialists were 1.18 and 3.65 times those of primary care physicians, respectively (13). We used the Consumer Price Indices (CPI) for medical care to adjust the 1999 payments for inflation through the year 2002 (14).

Table 1. Costs[a] of inpatient treatment, by treatment/service category, for 119 patients with West Nile virus illness, Louisiana, 2002

Treatment/Service category	n[b]	Per patient costs ($)			Total costs ($)
		Median	Range	Interquartile range	
Pharmacy/Medical supplies	115	2,934	16–88,825	994–7,601	887,759
Diagnostic	118	2,417	95–42,064	1,370–4,844	547,935
Room and board	117	1,132	52–16,445	640–2,266	237,917
Medical/Surgical services	75	675	15–21,606	183–3,261	200,233
Intensive care	24	5,526	439–17,769	2,320–11,001	162,360
Rehabilitation[c]	35	425	71–4,202	189–367	32,947
Emergency service	79	271	90–1,416	195–372	25,947
Other	33	109	1–2,620	20–247	8,873
Total	119	8,274	623–164,668	3,627–18,197	2,103,971

[a]Economic costs were estimated on the basis of hospital charges, by using Medicare cost-to-charge ratio for Louisiana—0.41 for urban areas and 0.488 for rural areas.
[b]n, number of patients who incurred costs in this category among the 119 patients. Per patient statistics include only the patients who incurred costs in this category.
[c]Rehabilitation costs indicated are those incurred during acute care hospital treatment. Costs of rehabilitation treatment in rehabilitation facilities are not included.

Charges for outpatient treatment in hospitals were available for 50 patients. Although outpatient treatment costs for hospitalized and nonhospitalized patients might be different, the available data could not be separated and thus the outpatient costs that were estimated based on the combined data for hospitalized and nonhospitalized patients were extrapolated to all reported WNV cases.

Although 60% of the questionnaire respondents indicated outpatient medication expenses, these respondents could not accurately recall the names and amounts of medications taken. Therefore, we did not include outpatient medication costs in the total cost of the outbreak.

The questionnaire was used to gather information about durable medical equipment use. Equipment costs were estimated on the basis of the 1999 MedStat Marketscan database data (Appendix 3) and adjusted to 2002 dollars by using a CPI medical care component. Assuming that the patients for whom durable equipment data were available (139 questionnaire respondents) were representative of all 204 CNS patients, we extrapolated the costs to all CNS patients requiring durable equipment.

Nonmedical Costs

Productivity Losses Attributable to Illness and Death

We used the human-capital method to estimate productivity losses attributable to illness and death (10). The productivity losses are measured as income forgone because of illness or premature death. These losses are also referred to as mortality cost. Information about workdays missed by patients or caregivers was obtained through the patient questionnaire. Of 139 respondents, 65 were employed before becoming infected with WNV. Respondents provided information about their earnings; income data were missing for 12 of 65 patients and for 15 of 36 caregivers who missed work to care for a patient. For these cases we used Bureau of Labor Statistics 2001 Louisiana state occupational employment and wage estimates (15), converted to 2002 dollars using the ratio of 2002 hourly wages to 2001 hourly wages (16). Ten of these 65 patients reported stopping work entirely because of WNV illness. Because the dates when each stopped working were not available and when each could resume work was unknown, we estimated their productivity losses from the second week of August (about half of Louisiana cases occurred before this date) until the last patient interview on February 27, 2003. Respondents provided information on their earnings before they stopped working. We estimated their productivity losses with the methods described.

The Louisiana Office of Public Health provided demographic information about patients who died. For persons $\leq$75 years of age, mortality costs were estimated as the present value of labor market earnings and household production based on productivity loss tables (17). Because the tables presented the current values of productivity losses at 5-year intervals, we interpolated the present values for consecutive ages within that 5-year interval and chose the values corresponding to deceased patients.

For persons 76–85 years of age, we used productivity loss tables on the annual weighted average earnings (1990 dollars adjusted to 2002 dollars [16]) by age group for labor-force and nonlabor-force persons (18). We estimated the expected lifespan for each age group using life tables for the total U.S. population (19) (Appendix 4). Then we added the annual earnings by age throughout the expected lifespan of the person, while making adjustments for a 3% discount rate (defined in Appendix 2) to calculate the present value of the person's earnings during future years of his or her lifespan (3% discount rate is recommended by the U.S. Public Health Service Panel on Cost-Effectiveness in Health and Medicine [20]) and a 1% annual productivity increase (1% is the usual assumption for long-term growth in labor productivity [17]).

Nursing Home, Transportation, and Miscellaneous Costs

Information about nursing home admissions and length of stay because of WNV illness was obtained through the patient questionnaire. We used the average daily cost for nursing homes in Louisiana from General Electric's long-term care insurance data (21).

The questionnaire obtained information on the frequency of doctors' visits and the distance that patients had to travel to see a physician. Transportation costs were estimated by using the U.S. federal government reimbursement rate of 36.5 cents per mile (22). Information about payments made for home health aides and miscellaneous services, such as cleaning, garden work, or babysitting, was obtained through the questionnaire.

Costs of Public Agencies

The Louisiana Office of Public Health incurred costs for laboratory support (human serum processing, diagnostic tests), epidemiologic aid (assessment of vector mosquito populations, active surveillance), administrative and clerical activities, and communication services. Information on these expenses was provided by the Louisiana Office of Public Health central office. State and local governments incurred costs for emergency vector control. Only expenditures resulting from the 2002 WNV epidemic in Louisiana that were above and beyond normal operating expenses were attributed to the WNV epidemic.

The core document used to estimate mosquito control program costs in Louisiana was the Louisiana Office of Emergency Preparedness summary of state reimbursement requests by 93 entities, such as mosquito abatement pro-

grams, parish police juries (parishes in Louisiana correspond to counties, and police juries to county boards of commissioners or similar local governing bodies in other states), and city governments. Expenses eligible for state reimbursement were for overtime labor, rented equipment, and materials exceeding normal budget expenses from June 1 to mid-August 2002. This amount, however, did not cover "payment-in-kind" activities, such as unpaid overtime, the transfer of employees from one activity to another, and replacement or repair of existing equipment extensively used during the epidemic. Many mosquito control units continued WNV control activities from mid-August until November. After November, we gathered from 18 mosquito control units and local government offices an updated estimate of all expenses incurred in 2002 attributable to the WNV epidemic. The ratio of reimbursement requests to total estimated expenses for these 18 entities was 1:1.7. The total requested state reimbursement amount for the 93 entities was multiplied by this ratio to get an estimate of the total expenses for mosquito control attributable to the WNV epidemic in Louisiana. No data were available to allow us to sample the entities by their size or scope of operation. Therefore, the mosquito control programs and local governments that responded to our inquiries may not have been representative of all the entities.

Results

The source of data and the estimated number of cases that incurred costs in each cost category are presented in Table 2. A summary of all estimated costs for the 2002 Louisiana WNV epidemic is presented in Table 3. The total estimated cost of the WNV epidemic in Louisiana in 2002 was $20.14 million.

Medical Costs

Acute-Care Inpatient Costs and Inpatient Rehabilitation Costs

We received information about acute-care hospital inpatient charges for 119 patients. Total charges for these 119 patients were $5.1 million, from which we estimated an economic cost of $2.1 million (the median cost per patient was $8,274, with a range of $623–$164,688) (Table 1). The economic costs for 71 (60%) patients were <$10,000 (Figure). If we assume that the total number of hospitalized patients with WNV in Louisiana was equal to the number of CNS illness cases, the estimated total costs of inpatient hospitalization were $3.6 million for the 204 CNS illness patients.

The median hospital stay was 8 days, with a median of 7 days for intensive care (Table 4). The daily median costs of stay were $160 for a standard room (range $98–$392), $537 for the intensive care unit (range $220–$1226), and $249 for the intermediate, post–intensive care unit (range $161–$314).

Nineteen (14%) of 139 survey respondents received inpatient treatment at a rehabilitation facility. Hospital charges were available for seven patients; the total cost for inpatient rehabilitation treatment for those seven patients was $96,556. Using the methods described in Appendix 1,

Table 2. Source of data and number of West Nile virus (WNV) cases for which data were available and estimated number of all cases that incurred costs in a given cost category, Louisiana, 2002

Cost category	Source	$N_{available}$[a]	$N_{cost\text{-}available}$[b]	N_{total}[c]	$N_{cost\text{-}total}$[d]
Inpatient					
Acute care	12 hospitals with >3 adult patients	119	119	204	204
Rehabilitation facilities	Patient's survey	139	19[e]	204	28
Outpatient					
Hospital care	6 of 12 hospitals	159[f]	50	329	103
Doctors' visits	Patient's survey	139	119	329	282
Rehabilitation facilities	Patient's survey	139	43	204	63
Durable equipment	Patient's survey	139	36	204	54
Productivity losses					
Mortality	Louisiana Office of Public Health data	24	NA[g]	NA	NA
Morbidity	Patient survey	139	99	204	146
Nursing home	Patient survey	139	5	204	7
Transportation	Patient survey	139	139	329	329

[a]$N_{available}$, number of patients for whom data were available from the indicated source, including cases with zero costs.
[b]$N_{cost\text{-}available}$, number of patients who incurred costs in that particular category out of all patients for whom data were available.
[c]N_{total}, number of patients who potentially could have incurred costs in the given cost category; for example, the number of all WNV cases in Louisiana, or the number of total central nervous system cases in Louisiana.
[d]$N_{cost\text{-}total}$ is estimated by the methods described in Appendix 1.
[e]The cost of inpatient rehabilitation treatment was estimated based on seven patients' charges received from four acute-care hospital-based rehabilitation centers.
[f]Of the159 patients for whom hospital charges were available from the 12 acute care hospitals, 50 from 6 hospitals incurred costs for outpatient hospital treatment.
[g]Not applied.

Table 3. Summary of costs attributable to 2002 West Nile virus epidemic in Louisiana

Cost category	Cost ($ millions)	% of total epidemic cost
Medical Costs		
Acute care	3.60	
Inpatient rehabilitation facility	0.39	
Inpatient treatment subtotal	**3.99**	20
Outpatient hospital care	0.03	
Visits to medical doctors	0.13	
Outpatient rehabilitation facilities, equipment	0.25	
Outpatient treatment subtotal	**0.41**	2
Total medical costs	**4.39**	22
Nonmedical Costs		
Mortality	5.40	
Morbidity	1.01	
Productivity losses subtotal	**6.41**	32
Nursing home	0.05	
Transportation, miscellaneous	0.09	
Total nonmedical costs	**6.55**	33
Total cost of illness	***10.94***	***54***
Mosquito control	8.30	
Louisiana Office of Public Health	0.90	
Total public agency cost	***9.20***	***46***
Total cost of epidemic	***20.14***	***100***

we estimated that 28 of 204 CNS case-patients in Louisiana received inpatient rehabilitation treatment at a total cost of $386,000.

Costs of Outpatient Hospital Treatment and Physician Visits

Of 159 patients for whom hospital charges were available, 50 (32%) received outpatient hospital treatment at a total cost of $14,539. Using these numbers, we estimated that 103 of 329 persons reported to the Louisiana Office of Public Health received outpatient hospital treatment, at an estimated cost of $30,000. The estimated total cost of visits to see a primary care doctor, specialist, or both for 139 patients who responded to the survey was $54,572; extrapolating this figure to the 329 reported WNV cases yielded an estimated cost of $129,000.

Costs of Outpatient Rehabilitation Therapies and Durable Medical Equipment

Thirty-one (22%) of 139 respondents reported receiving outpatient physical therapy, with an estimated cost of $110,184. Ten of 139 patients reported receiving occupational therapy, with a total estimated cost of $35,207. Two patients received speech therapy, at a total estimated cost of $1,025. The total estimated cost for outpatient rehabilitation therapy for these 139 survey respondents was $146,417; extrapolating this figure to 204 CNS case-patients in Louisiana yielded an estimated cost of $215,000. The cost of durable medical equipment (36 of 139 respondents used medical durable equipment such as a wheelchair, walker, cane, breathing treatment machine, treadmill, and hospital bed) extrapolated to the 204 CNS case-patients was an estimated $31,000.

Nonmedical Costs

Productivity Losses from Illness and Death

For 53 patients who missed work but did not stop working entirely, the estimated productivity losses were $443,000 (the average number of days missed was 50, and the median number of days missed was 37, with a range of 1 to 212 days). Extrapolating this figure to 204 CNS patients, we estimated that 78 missed work, at a total productivity loss of $652,000. For the 10 patients who stopped working entirely, the estimated productivity losses were $157,950. Based on these data, we estimated that 15 of 204 CNS patients stopped working entirely, at a total cost of $237,000 (Appendix 1). Thirty-six of 139 respondents indicated that someone missed work to take care of them; the resulting productivity loss totaled $82,669 dollars. The extrapolated cost for caregivers for the 204 CNS patients was $122,000. The total extrapolated illness cost attributable to WNV infection was $1.01 million.

Twenty-four deaths were attributed to WNV illness in Louisiana in 2002. The median age of deceased patients was 78 (range 27–94). The total estimated mortality cost for these 24 persons was $5.4 million, which was >50% of the illness-associated costs and >25% of the total costs of the epidemic.

Nursing Home, Transportation, and Miscellaneous Costs

Five (4%) of 139 patients 45–86 years of age were reported to have spent 21–170 days in a nursing home

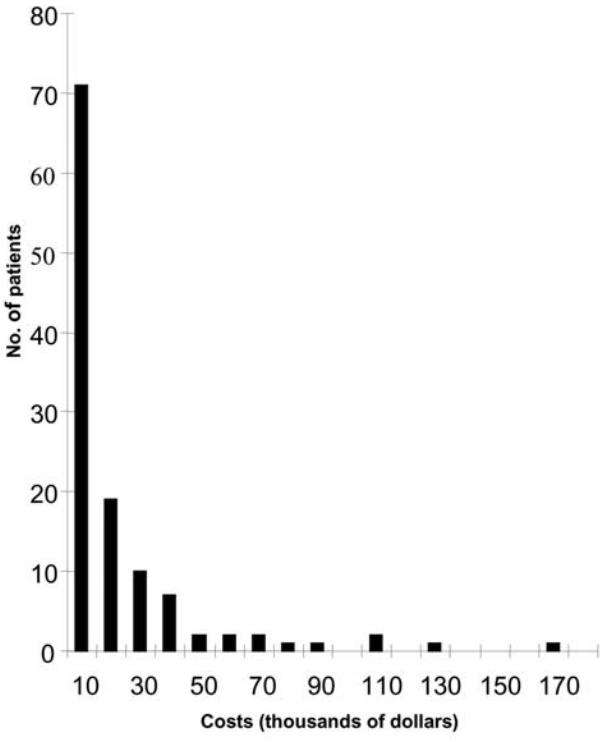

Figure. Number of hospitalized patients (N = 119) with West Nile virus infection, by cost of inpatient treatment; Louisiana, 2002.

because of complications from WNV infection. Two of these patients remained in a nursing home at the time of their interviews in December 2002 and February 2003. The estimated total payment for nursing home care for the five patients was $36,956; the total estimated nursing home costs for Louisiana CNS patients were $54,000.

The estimated transportation cost for 139 respondents was $8,354. If one assumes that the transportation costs for the 139 respondents were representative of costs for those who did not participate in our survey, the estimated total cost of transportation for the 329 WNV cases was $20,000.

Twenty survey respondents reported having used home health aides or other services, such as babysitting, house cleaning, or yard work, at a reported total cost of $29,225. When this figure is extrapolated to the 329 WNV cases in Louisiana, miscellaneous expenses were at least $69,000.

Costs of Public Agencies

Mosquito Surveillance and Abatement

From June 1 to mid-August, 2002, a total of 93 public offices requested $4,879,070 as state reimbursement from the Louisiana Office of Emergency Preparedness. Eighteen mosquito control units and local government offices reported their estimated total expenses. Using the ratio of the sums of the requested reimbursement amounts to the total reported expenses of $1:$1.7, we estimated that the cost of mosquito surveillance and abatement programs for these 93 entities was $8.3 million.

Public Health Office Costs

From June to November 2002, the central state public health office incurred an estimated $886,000 in expenses because of WNV. From this total amount, basic operating expenses cost $586,000, contracts such as for veterinary diagnostic and entomologic services cost $166,000, and laboratory expenses cost $134,000.

Discussion

We estimated that the costs from June 2002 to February 2003 attributable to the 2002 WNV epidemic in Louisiana were $20.1 million (Table 3). This figure is likely an underestimate since some of the costs associated with illness or public health response were not available, such as costs for outpatient medication and costs incurred by persons with WNV infections who were not identified or reported to Louisiana Office of Public Health. Long-term costs of WNV illness sequelae were not evaluated.

Although the costs of medical care, wages, and cost of living vary by region, we assumed that the Louisiana costs were representative of those elsewhere in order to roughly estimate the magnitude of the WNV epidemic nationwide. Extrapolating to the 4,156 cases (2,942 CNS cases) reported nationwide, the short-term costs of inpatient treatment would be $57.5 million, outpatient treatment costs would be $5.6 million, and nonmedical costs would be $76.7 million, for a sum of $139.8 million. This figure does not include mosquito abatement and prevention costs (mosquito control capabilities vary tremendously from state to state), which accounted for approximately half of the costs in Louisiana.

To our knowledge, only a study of the 1966 St. Louis encephalitis virus epidemic in Dallas, Texas (172 cases, 20 deaths), estimated the cost of a mosquitoborne disease epidemic in the United States (4). The total costs of that epidemic were an estimated $796,500 in 1966 dollars. Adjusting each cost component by the appropriate CPI (using CPI for all items or for medical care), the total epidemic cost was $5.4 million in 2002 dollars, from which the largest share was for epidemic control expenditures ($348,500 in 1966 dollars [$1.9 million in 2002 dollars]).

The time frame of our study was from June 2002, when the epidemic was first recognized, until the last date we administered the survey, February 2003, some 3 months after the onset of illness of the last reported case-patient. Several patients, however, likely incurred further costs beyond the date of their interview. Seventy-three (53%) of 139 survey respondents indicated that they expected to get

Table 4. Number of hospital days for 119 patients with West Nile virus illness, Louisiana, 2002

	n[a]	Mean	Median	Range	Interquartile range
Regular room	117	9	7	1–62	4–10
Intensive care	24	11	7	2–29	4–17
Post–intensive care, intermediate intensive care	8	5	3	1–11	2–9
Any room	119	12	8	1–76	4–13

[a]n, number of patients of the 119 patients who incurred costs in a given category.

further treatment because of health problems caused by WNV. When, or if, those who stopped working will be able to resume work is also unknown. Another limitation of our study was the possible bias in the estimations that were based on the information gathered by the patient survey. We did not have the information to determine whether differences existed between the nonrespondent and respondent groups.

Although the future incidence of WNV disease cannot be predicted, WNV incidence will likely remain substantially greater than the total incidences of arbovirus infections previously known to be endemic to the United States (23). These Louisiana data suggest that even short-term costs attributable to WNV epidemics are substantial.

The costs associated with WNV epidemics such as those documented here can be used to evaluate the economics of WNV prevention and control programs. To fully evaluate the economics of prevention programs, epidemiologic and mosquito control data related to program effectiveness are necessary.

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This study, protocol #3679, has been approved by the CDC Institutional Review Board/Human Subjects Activity.

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Appendix 1. Extrapolation Methods

If we assume that the case-patients for whom data were available were representative of case-patients for whom data were not available, to estimate the total cost of all applicable cases, C_{total}, first we estimated the total number of cases who would have incurred costs in that particular cost category, $N_{cost\text{-}total}$.

$$N_{cost-total} = N_{cost-available} \times \frac{N_{total}}{N_{available}}$$

$N_{cost\text{-}available}$ indicates the number of case-patients who incurred costs in that particular category out of all case-patients for whom data were available, $N_{available}$, which also includes case-patients with zero costs. N_{total} is the number of all case-patients who potentially could have incurred costs in that cost category, for example, the number of total WNV case-patients in Louisiana, or the number of total central nervous system case-patients infected with WNV in Louisiana. The total cost for a given cost category would be:

$$C_{total} = C_{available} \times \frac{N_{cost-total}}{N_{cost-available}}$$

where $C_{available}$ is the cost for available cases.

All extrapolated estimates presented in the paper are rounded to their nearest $1,000.

Appendix 2. Explanation of Economic Terms for Noneconomists

Economic (True) Cost and Cost-to-Charge Ratios

Economic (true) cost means opportunity cost of a resource. Economists are usually interested in societal costs of health programs—the value of benefits that would have been derived if the resources had been allocated to their next best use, i.e., the opportunity cost of resources. In perfect markets (explanation of costs in perfect markets follows the definition of cost-to-charge ratios), the market prices of resources reflect their opportunity costs. Because of healthcare market imperfections (explained below), charges made by healthcare providers do not usually reflect opportunity cost and are generally higher than the cost of resources used (explanation for the reasons of charges being higher than costs in health care is provided in this appendix under subtitle Asymmetric Information). Large insurance companies and the government (Medicare/Medicaid) reimburse hospitals and physicians at a much lower rate than the charges made by the healthcare provider. These reimbursements are closer to the actual costs of the resources used than the charges made by the healthcare provider.

The common method for estimating the true economic cost of medical services is adjusting the charges through the use of "cost-to-charge ratios." Cost-to-charge ratios are coefficients developed by expert panels to convert charges for medical services to their true economic costs. They represent an average estimate of true costs. The Federal Register publishes state by state Medicare cost-to-charge ratios every year. The ratios are different for urban and rural areas.

Costs in Perfect Markets

Obtaining the opportunity cost of a resource is difficult. In perfect markets, the market prices of resources reflect their opportunity costs. Therefore, to determine opportunity costs, we have to collect market prices for goods traded in perfect markets. Perfect market conditions exist when 1) numerous buyers and sellers can enter and withdraw from the market at no cost, 2) all buyers are identical, 3) all buyers possess the same relevant information, and 4) goods and services traded are the same. In reality, one or many conditions of perfect markets are violated in most markets. Economists call them imperfect markets. Various methods are used to estimate the costs of resources when conditions for a perfect market are violated or the resources are not traded in the marketplace. Healthcare markets do not meet the conditions for perfect markets for a number of reasons, including those discussed in the following sections.

Asymmetric Information

Consumers in healthcare markets generally have little information about the treatments medical professionals offer them. They are at a disadvantage to make fully informed choices. Economists refer to such a difference in access to information between market participants as asymmetric information. Asymmetric information allows the sellers to charge prices for medical services that are higher than opportunity costs.

Market Power

The size and limited number of health insurance companies—the important participants in healthcare markets who "buy" care

from providers—gives them considerable market power to influence the prices of goods and services sold in that market. Health insurance companies representing large numbers of subscribers use their weight to negotiate discounts from hospitals and doctors (the "sellers"). Therefore, the prices paid to providers vary with the insurance status of patients and do not correspond to opportunity costs. For more details on economic costs and cost-to-charge ratio method, see Haddix et al. (1) or Meltzer (2).

Discount Rate

Discounting is an economic notion that, even in a world of zero inflation, a dollar today would be of higher value to a person than a dollar in the future. A dollar today can be used to purchase a good or service now instead of making the purchase later. This concept is referred to as time preference. The premium placed on benefits today versus the future is reflected in the rate at which a person is willing to exchange present for future costs and benefits. This quantitative measure of time preference is called the discount rate. When the costs or benefits under the study continue in the future, in order to make them comparable in terms of the time dimension economists calculate the present value of these costs or benefits by using discount rates. The U.S. Public Health Service Panel on Cost-Effectiveness in Health and Medicine recommends a 3% discount rate for economic studies in health (3).

Appendix 2 References

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Appendix 3. Estimating Costs of Durable Equipment

Costs vary among the types of similar durable equipment. For example, costs for different types of wheelchairs vary considerably. Because the particular type of equipment each patient used could not be accurately ascertained, we estimated the expected cost of that equipment on the basis of data available from the Marketscan database, which provided average national payments for each type of durable equipment. Let N_i be the number of payments reported to Marketscan for the i^{th} type of the equipment, where $i=1,2, \ldots n$. N is the total number of payments reported to Marketscan for all types of that durable equipment:

$$N = \sum_{i=1}^{n} N_i$$

C_i is the mean payment for the i^{th} type of equipment. For certain equipment, such as wheelchair, C_i might represent an average payment for rental of that equipment. Since we do not know the type of equipment the patient bought or rented, we view the relative frequency $\frac{N_i}{N}$ as the probability of a patient purchasing that particular type of equipment. We estimated the expected cost of the given durable equipment as:

$$EC = \sum_{i=1}^{n} C_i \times \frac{N_i}{N}$$

Appendix 4. Estimating Expected Life Years for a Person

The life table of the total U.S. population for the year 2000 provided numbers of survivors, by 1-year increments, from birth to a given age, starting with a cohort of 100,000 people (1). At each age, the expected life years for the surviving cohort was also provided. The expected life years for a person in our study, ELY_i, was estimated as the product of the person's survival rate and the expected life years for the cohort, ELY_{cohort}, where the survival rate for a person is equal to the ratio of the number of survivors until the expected age for the cohort, $N_{survivors}$, to the number of persons in the cohort at a given age, $N_{individuals}$:

$$ELY_i = \frac{N_{survivors}}{N_{individuals}} \times ELY_{cohort}$$

The estimated expected life years for a person 76 or 77 years of age were 5 years. For persons 78–81 years of age, the estimated individual life years were 4. For persons 82–84 years, the expected life years were 3. We assumed that for persons >85 years, the productivity losses were 0; therefore, the expected life years for persons ≥85 were not relevant in our application.

Appendix 4 Reference

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Virus-specific RNA and Antibody from Convalescent-phase SARS Patients Discharged from Hospital

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Severe acute respiratory syndrome (SARS) is caused by a novel coronavirus (SARS-CoV). In a longitudinal cross-sectional study, we determined the prevalence of virus in bodily excretions and time of seroconversion in discharged patients with SARS. Conjunctival, throat, stool, and urine specimens were collected weekly from 64 patients and tested for SARS-CoV RNA by real-time polymerase chain reaction; serum samples were collected weekly and tested for SARS-CoV antibody with indirect enzyme immunoassay and immunofluorescence assay. In total, 126 conjunctival, 124 throat swab, 116 stool, and 124 urine specimens were analyzed. Five patients had positive stool samples, collected in weeks 5–9. Two patients seroconverted in weeks 7 and 8; the others were seropositive at the first serum sample collection. In this study, 5 (7.8%) of 64 patients continued to shed viral RNA in stool samples only, for up to week 8 of illness. Most seroconversions occurred by week 6 of illness.

Severe acute respiratory syndrome (SARS), a newly-defined condition identified in March 2003, is caused by the novel SARS-associated coronavirus (SARS-CoV). It is believed to have originated in Guangdong Province, China, with subsequent spread to other cities and countries. By August 15, 2003, a total of 30 countries had reported cases of SARS; 5 countries had local transmission. Approximately 8,500 cases were reported and at least 916 deaths (1). Singapore's index case-patient was a 22-year-old woman admitted to the hospital for atypical pneumonia on March 1, 2003; she had returned from a trip to Hong Kong on February 25. An additional 237 cases were identified and 33 deaths. The date of onset of illness of the last patient was May 5, 2003.

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The role of body fluids in transmission and the potential for transmission during convalescence remain undefined. In Hong Kong, for example, faulty sewage works in a residential block were deemed to be the cause of a major outbreak in a residential estate (2). Excretion of the virus RNA in bodily fluids has also been reported during the convalescent phase (3), despite the patient's apparent clinical recovery. Detecting SARS-CoV in excreted bodily fluids would thus have substantial implications on infection control measures for recovered patients returning to the community.

The relationship of potential virus secretion to seroconversion is also undefined, as is the optimal time to test for seroconversion. Seroconversion has been suggested to occur at a mean of 20 days of illness (4). Recently available laboratory tests for detecting antibodies against SARS-CoV provide a means to answer these clinical questions.

A longitudinal cross-sectional study was designed to study the prevalence of the virus and the duration of viral shedding in bodily excretions (stool, urine, throat secretions, and conjunctival tears) by using nucleic acid amplification tests on samples from patients who had recently recovered from SARS. We also sought to determine the factors for viral shedding and the time of seroconversion in patients recovering from SARS.

Methods

Patients

Patients for the study were recruited at the Tan Tock Seng Hospital, Singapore, from April 30 to May 30, 2003. The government designated this hospital for all SARS-infected patients. A total of 233 of the 238 patients with

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reported probable cases of SARS in Singapore were cared for at this hospital. Approval for this study was obtained from the Ministry of Health, Singapore, and the local hospital ethics committee. Consent was obtained from all recruited patients.

Patients were identified from the existing patient registry maintained at the hospital. Selection criteria were a diagnosis of SARS based on current World Health Organization (WHO) recommendations (5) and being a discharged patient. Patients were recruited into the study anytime from the day of discharge up to 42 days after discharge. Discharge criteria were based on existing WHO recommendations (6). In our study, the endpoint of collection was arbitrarily set at 42 days after discharge. Given an estimated 14- to 21-day hospital stay for each patient, our study design would allow sampling up to days 56 to 63 of illness. During the initial study design, one report (3) showed viral RNA in stool samples from convalescent-phase patients up to day 25 of illness. Collection of specimens up to day 63 appeared sufficient. A dedicated team of four doctors collected weekly specimens from the throat and conjunctiva from each consenting patient. Fresh specimens of midstream urine, stool, and blood were collected concurrently in sterile specimen bottles.

Sample Collection

A sterile swab was used to collect throat secretions from the fornices and back of the throat. A spatula was used to depress the tongue during the procedure. The swab was then immediately placed in a bottle containing Hank's viral transport medium. By using a sterile swab, the conjunctiva of one lower eyelid was swabbed with one brush. The swab was then rotated 180°, and the conjunctiva of the other lower eyelid was then swabbed similarly. The swab was immediately placed in a bottle containing Hank's viral transport medium. Midstream urine was collected in a sterile urine container. Stool specimens were collected with a spatula into a sterile stool container. We accepted urine and stool specimens that were delivered within 12 hours of collection. Nine milliliters of blood was collected in tubes with gel serum separators and kept at 4°C. All specimens upon receipt, except blood, were kept at –70°C until processed. Weekly collections of all the specimens were repeated until 42 days after discharge. Information on demographics and clinical course was collected.

Laboratory Test Methods

Laboratory tests were performed at the Department of Pathology, Singapore General Hospital. Reverse transcription–polymerase chain reaction (RT-PCR) was performed on conjunctival, throat, stool, and urine specimens with the RealArt HPA-coronavirus RT-PCR kit (Artus Gmbh, Hamburg, Germany) on the Roche Lightcycler (Roche Diagnostics Corporation, Indianapolis, IN), a real-time PCR instrument. This assay targeted the polymerase gene of SARS-CoV. All positive results were verified, with two other primer pairs also targeted at the polymerase gene of SARS-CoV. The first were primers designed by the Genome Institute of Singapore (7), the second were Cor 1/2 primers from the Government Virus Unit, Hong Kong (sense 5′ CAC CGT TTC TAC AGG TTA GCT AAC GA 3′, antisense 5′ AAA TGT TTA CGC AGG TAA GCG TAA AA 3′) (8). Detection limit was set at 1 copy/μL of specimen. Serum specimens were tested for total virus-specific antibodies with an indirect enzyme immunoassay (EIA) with SARS-CoV lysate as the antigen (9). Positive serum samples were retested for immunoglobulin (Ig) G by immunofluorescence assay (IFA) with SARS-CoV–infected Vero cells spotted onto microscope slides.

Statistical Analysis

During recruitment, patients were selected on the basis of the number of days after the patient was discharged from the hospital. During analysis, this date was converted to day of illness, and subsequently week of illness, by determining the date of onset of illness. Onset of illness was defined as the day of onset of fever. For example, specimens collected from day 15 to 21 of illness were thus grouped into week 3 of onset of illness. Statistical analyses were performed with SPSS for Windows release 10.0.1 (SPSS, Inc., Chicago, IL). Categorical variables were analyzed with chi-square test. The student *t* test was used to analyze continuous variables.

Results

Patients

A total of 170 patients met the selection criteria, 64 patients consented to the study, and 4 patients withdrew after the first collection. The mean age of consenting patients was 35.2 years (range 17–63 years), and 25% were men. The mean age of all SARS patients in Singapore was 35 years (range 1.3– 90 years), and 32.4% were male. Patients for this study were recruited from week 3 to 9 of their illness (median week 7) (Figure 1). Most patients (89%) were recruited after week 4 of illness.

Ten participants had coexisting conditions (diabetes mellitus, hypertension, asthma). Four had a history of previous intensive care admission. During their hospital stay, six had history of steroid treatment, defined as prior use of hydrocortisone, prednisolone, dexamethasone, or pulsed methylprednisolone, regardless of dosage and frequency. Ribavirin was used in the treatment of 32 patients (50%).

Laboratory Investigations

Since an interim analysis on 60 specimens indicated no

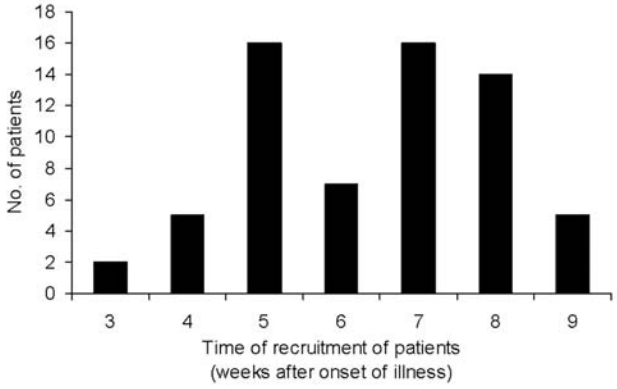

Figure 1. Recruitment of patients by week of illness.

positive yields 14 days after discharge, a decision was made to stop all subsequent collections until after day 28 of discharge. Specimens already collected were processed.

In total, 126 conjunctival specimens, 124 throat swab specimens, 116 stool specimens, 124 urine specimens, and 123 blood specimens were available for analysis. An average of 9.6 specimens was taken per patient (range 4–19). All patients had at least one specimen of each type taken.

RT-PCR

Most (76%) specimens collected for RT-PCR testing were taken from week 6 to 8 of illness (mean 6.3 weeks, standard deviation [SD] 1.47). All specimens from throat, conjunctiva, and urine were negative for SARS-CoV by RT-PCR. Six stool specimens from five patients were positive by the three RT-PCR assays (Figure 2).

Relevant clinical histories and laboratory data for these patients are summarized in the Table. The five patients with positive viral RNA in stool specimens had a mean age of 31.6 years; two (40%) of the five patients required supplemental oxygen. No patients were admitted to intensive care. Mean peak lactate dehydrogenase (LDH) was 811 U/L. The 59 patients with stool samples negative for SARS-CoV RNA had a mean age of 35.9 years, 33% required supplemental oxygen, and 4 were previously admitted to intensive care. Mean peak LDH for the patients without RNA detected in stool samples was 764 U/L. Age, prior supplemental oxygen use, and peak LDH were not statistically different in the two groups.

Patients 3 and 62 provided one sample each and withdrew from the study immediately thereafter. Both were positive for SARS-CoV by RT-PCR in week 5 of illness. No subsequent specimens were available for analysis. Three stool specimens were taken from patient 16 on weeks 4–6 of illness. Only specimens taken in week 4 were positive. Patient 51 provided two stool specimens for week 5 and 6; both were positive. The sample taken in week 6 had fewer copies per reaction on RT-PCR compared to that taken in week 5. A sample requested by her physician, outside this study, was collected on week 10 of illness and tested negative by RT-PCR. Patient 35 provided one sample, which was positive, at week 9 of illness. No subsequent specimens were available.

Of the five patients with positive SARS-CoV detected by RT-PCR in the stool sample, one (patient 3) had a history of prior steroid treatment during SARS illness. As mentioned earlier, this patient provided one sample before withdrawing from the study. Five other patients received steroid treatment during their hospitalization, but none had SARS-CoV detected by RT-PCR. These patients were recruited from week 5 to 7 of illness.

Of the 32 patients who received ribavirin, two had samples positive for SARS-CoV RNA. In comparison, 3 of the 32 patients who did not receive ribavirin had SARS-CoV detected by RT-PCR. This difference was not significant. Diarrhea was reported in 19 (30%) of our study patients; one had a sample positive for SARS-CoV RNA. The remaining RNA-positive samples were from patients who did not report any diarrhea symptoms during their hospital stay. Ten of our participants had a history of chronic illnesses. Three had asthma, five had hypertension, and one had diabetes mellitus type 2. One additional patient had diabetes mellitus type 2 and hypertension. Samples were collected from these patients on weeks 5 to 9 of illness (mean 6.3 weeks [SD 1.22]). None of these patients had SARS-CoV viral RNA detected in our study.

Serologic Testing

A total of 123 specimens from 64 patients were available for analysis. All specimens were positive by EIA, with the exception of two specimens taken at week 7 and 6 from patients 4 and 5, respectively. All positive results were corroborated with a positive IFA, except for a sample from patient 38, which was negative by IFA on week 5 of illness. The next sample taken on week 6 was positive on both EIA and IFA. The result of the first sample of blood taken for serologic testing was plotted against the respective week of illness (Figure 3). For patients 5 and 4, whose initial results were negative, serologic test results became positive at the subsequent week of testing (weeks 7 and 8, respectively)

Discussion

SARS-CoV detected in mucosal secretions or excreta will affect decisions about discharging patients. Detecting SARS-CoV would have a greater importance if it was detected in the respiratory tract (throat swabs) or tears (conjunctival swabs). By testing samples from patients who were discharged from the hospital, we accessed patients deemed clinically fit to return to the community. A positive RT-PCR result in these patients would be more

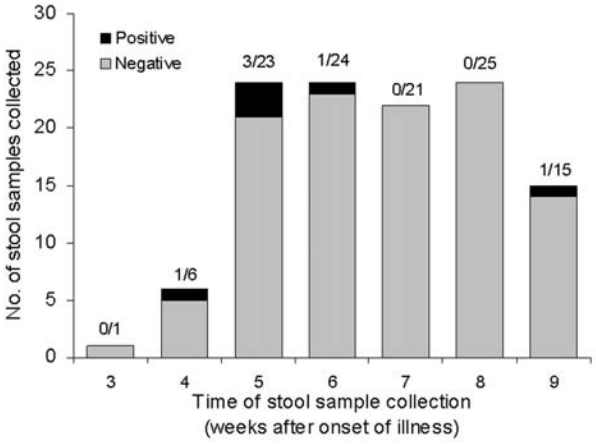

Figure 2. Results of severe acute respiratory syndrome–associated coronavirus polymerase chain reaction (PCR) on stool samples plotted by week of illness. Samples were processed for reverse transcription (RT)-PCR. Any result was deemed positive if it was detected by both the RealArt HPA-coronavirus RT-PCR kit (Artus Gmbh, Hamburg, Germany) and two other RT-PCR primers designed by Genome Institute of Singapore and the Government Virus Unit, Hong Kong.

important compared to results of those patients recovering in the hospital. Weekly tests were conducted to assess this risk by using molecular diagnostic methods. This is the first longitudinal cross-sectional study of viral shedding in convalescent-phase SARS patients.

By performing weekly tests, the optimal time of seroconversion in recovering patients could be determined. This information will assist clinicians in planning a reasonable time for serologic testing in patients with suspected cases of SARS.

In our study, viral nucleic acid for SARS-CoV was detected only in the stool samples. This finding was infrequent; most patients (>95%) did not have positive samples after week 5 of illness (Figure 2). One patient still had detectable viral RNA at week 9 of illness. None of our patients had viral nucleic acid for SARS-CoV detected in respiratory, urine, or conjunctival samples.

Detection of viral nucleic acid for SARS-CoV in stool samples of recovering patients has been reported. Samples were positive up to day 25 in a study by Drosten et al. (3) and at day 73 in a study by Leung et al. (10). The Drosten et al. study involved two patients; both had viral RNA detected during the convalescent phase on day 19 and 25 of illness. In the Leung et al. study, the authors examined the gastrointestinal manifestations of the 138 patients with SARS who were admitted to a local hospital. The overall detection rate of SARS-CoV RNA in stool specimens was 16%. Whether these samples were taken during the acute phase, convalescent phase, or both is unknown. The authors observed that in one patient, viral RNA was detected in a stool sample up to 73 days after symptom onset. No other clinical information was available for this patient.

In a later study, Chan et al. (11) reported that viral RNA was detected in ≈5% of stool samples submitted after week 7 of illness. The last positive sample was detected at week 11 of illness; in that study, the respiratory samples (tracheal aspirates, nasopharyngeal aspirates, throat swabs, throat washings, nasal swabs, and pooled throat and nasal swabs) were detected at week 8 of illness. All those who shed virus for a prolonged period (arbitrarily defined as >6 weeks after onset of symptoms) had samples collected while still critically ill. This study had a different cohort of patients compared to ours. Our patients had been discharged and had comparatively shorter and milder illnesses. Determining whether positive carriage persisted in these critically ill patients from the Chan et al. study after discharg would be beneficial.

SARS-CoV was not isolated in this study, so we were unable to assess if those with positive stool samples were infectious. However, in the Chan et al. study (11), 2 (1%) of the 195 samples submitted for stool isolation were

Table. Clinical and laboratory parameters of patients with stool samples positive for SARS-CoV RNA[a]

Patient no.	Age	Sex	Ethnicity	Occupation	Specimens provided	Viral load[b] (copies/mL)	History of ICU admission	Required supplemental oxygen	Peak LDH U/L	Concurrent illness
3	43	Male	Malay	Unemployed	Wk 5[c]	Wk 5 (33.9 x 10^3)	No	No	299	Diabetes mellitus type 2 Steroid use
16	33	Female	Indian	Domestic maid	Wks 4,[c] 5,6	Wk 4 (37.1 x 10^3)	No	Yes, I/N	1,127	None
35	17	Male	Chinese	Student	Wk 9[c]	Wk 9 (1.73 x 10^3)	No	No	615	None
51	30	Female	Filipino	Healthcare worker	Wks 5,[c] 6[c]	Wk 5 (2 x 10^3) Wk 6 (1.64 x 10^3)	No	No	1,065	None
62	35	Male	Chinese	Diver	Wk 5[c]	Wk 5 (7.76 x 10^3)	No	Yes, I/N	952	None

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus; ICU, intensive care unit; LDH, lactate dehydrogenase; I/N, intranasal oxygen.
[b]RT-PCR was performed by using the RealArt HPA-coronavirus RT-PCR kit (Artus Gmbh, Hamburg, Germany) on the Roche Lightcycler. Viral load was determined for each mL of stool sample.
[c]Positive RT-PCR results for SARS-CoV.

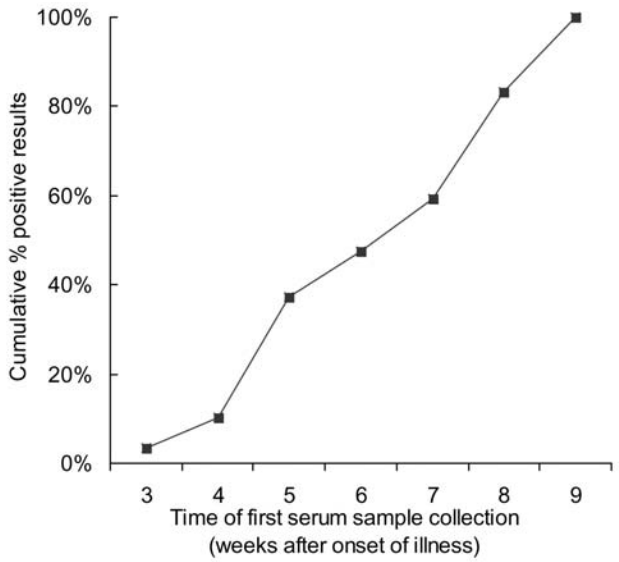

Figure 3. Cumulative results of first serologic testing of samples by week of illness. Serologic testing was performed by indirect enzyme immunoassay with severe acute respiratory syndrome–associated coronavirus lysate as the antigen.

positive, only during week 1 of illness. In the study by Leung et al. (10), virus was not isolated from stool samples. This finding was similar to our own experience in Singapore. Our laboratory was the primary center for SARS-CoV isolation during the epidemic, and we had not been successful in isolating viruses from stool samples. A stool sample positive for SARS-CoV, from convalescent-phase SARS patients, would probably have a minimal effect on the public. Secondary SARS cases from convalescent-phase SARS patients discharged from the hospital have not been reported. We, however, still recommend caution, especially in pediatric patients and adults requiring full nursing aid, since SARS-CoV can survive for at least 2 to 4 days at room temperature (12).

With a greater severity of illness, excretion of the virus would likely be prolonged. We identified severity of illness by a history of supplemental oxygen use, admission to intensive care, or a high peak LDH. Peak LDH has been identified along with advanced age as predictors of adverse outcomes in Hong Kong (13,14). However, in our study patients, age, supplemental oxygen use, and peak LDH were not statistically different between those who had positive stool samples and those who had negative stool samples. Samples from all four patients with a previous history of intensive care admission were negative for SARS-CoV RNA by RT-PCR. This finding suggests that the duration of viral RNA excretion in the convalescent phase may not be solely determined by severity of illness. However, the number of intensive care patients in the study was small.

Six patients (9.4%) in our study received steroid treatment compared to 17.2% of the SARS cohort in Singapore. Stool samples from one patient were positive for RNA. Possible explanations for this finding include the small numbers of those treated with steroids in our study and the fact that all those who had stool samples negative for RNA who were on steroids were recruited late in their illness (weeks 5–7).

SARS-CoV viral RNA was not detected in stool samples from the 10 patients with chronic illnesses. All those who did have SARS-CoV viral RNA had no history of chronic illnesses. Again, a possible explanation is the recruitment of patients late in their illness.

The use of ribavirin did not appear to reduce the detection rates of viral RNA in the stool sample. This finding concurred with observations from in vitro studies (15) and retrospective cohort studies (16), which suggested that ribavirin was not effective against SARS-CoV. In a separate study, Leong et al. (17) showed that ribavirin did not confer any survival benefit for patients with SARS.

Active replication of SARS-CoV has been reported in the small and large intestine (10). Diarrhea was not associated with a higher proportion of patients with SARS-CoV–positive stool samples. The increased intestinal motility caused by diarrhea might lessen the viral load in the gastrointestinal tract in those patients with symptoms.

In Hong Kong (4), IgG seroconversion was detected from day 10 of illness (mean of 20 days). Ninety-three percent of patients seroconverted by day 30 (week 5) of illness. As part of the protocol, all patients received steroids (intravenous hydrocortisone, oral prednisolone, or pulse methylprednisolone) on diagnosis of SARS. Steroids did not appear to affect seroconversion rates. The Toronto group (18) reported similar results. Seropositivity rate was 96.2% at 28 days of illness and beyond.

All but two of our patients demonstrated positive EIA results at the initial specimen collection. These results were expected, since most patients were recruited after week 4 of illness. At week 6 of illness, 27 (96%) of 28 patients had a positive serologic result. Patients 5 and 4 demonstrated seroconversion at week 7 and 8 of illness, respectively. This finding is congruent with the Hong Kong study; therefore, the diagnostic role of serologic testing is limited in the acute phase of illness.

Steroid use was infrequent (6 patients) in our study. Patient 5 received intravenous hydrocortisone during her hospital stay. The other five participants who received steroids during their hospital stay had detectable antibodies at the first specimen collection, performed at week 5 (four patients) and week 7 (one patient). Patient 4 seroconverted at week 8, and she had no prior steroid use, which suggests that the serologic response is not muted by the use of steroids. The two patients who seroconverted during these sequential weekly collections had ribavirin and symptoms of diarrhea during their hospital stay.

Our study had limiting factors. The design of this study was cross-sectional, which did not allow us to accurately determine when seroconversion occurred or when viral RNA ceased to be detectable. Most of our patients did not have severe illness, and most intensive care patients declined to participate. Returning to the hospital on a weekly basis for specimen sampling was also difficult because these patients had substantial sequelae from the infection. Even when recruited, participants were already in the later half of their convalescence. These patients have had more severe illness and would have had a longer hospital stay. At the time of discharge, they would have been beyond week 4 of illness. Our study had few participants in the early phase of recovery. The outbreak in Singapore was already under control when the study commenced at the end of April, which further limited the number of eligible participants in the early convalescent phase.

Conclusion

Detection of SARS-CoV RNA is uncommon in recovering patients discharged from the hospital. In this study, RT-PCR determined that 5 (7.8%) of 64 patients continued to shed viral RNA in stool samples only, up to week 8 of illness. Most seroconversions (96%) occurred by week 6 of illness, although seroconversion may still occur at week 7 and 8 of illness. Excretion of viral RNA and seroconversion did not appear to be related to age, underlying conditions, diarrhea, prior steroid or ribavirin use, or severity of illness. No secondary cases of infection occurred among the convalescent-phase patients.

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Fluoroquinolone Resistance in Penicillin-resistant *Streptococcus pneumoniae* Clones, Spain

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Among 2,882 *Streptococcus pneumoniae* sent to the Spanish Reference Laboratory during 2002, 75 (2.6%) were ciprofloxacin-resistant. Resistance was associated with older patients (3.9% in adults and 7.2% in patients ≥65 years of age), with isolation from noninvasive sites (4.3% vs. 1.0%), and with penicillin and macrolide resistance. Among 14 low-level resistant (MIC 4–8 μg/mL) strains, 1 had a fluoroquinolone efflux phenotype, and 13 showed single ParC changes. The 61 high-level ciprofloxacin-resistant (MIC ≥16 μg/mL) strains showed either two or three changes at ParC, ParE, and GyrA. Resistance was acquired either by point mutation (70 strains) or by recombination with viridans streptococci (4 strains) at the topoisomerase II genes. Although 36 pulsed-field gel electrophoresis patterns were observed, 5 international multiresistant clones (Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, Spain^{14}-5 and Sweden^{15A}-25) accounted for 35 (46.7%) of the ciprofloxacin-resistant strains. Continuous surveillance is needed to prevent the dissemination of these clones.

Resistance of *Streptococcus pneumoniae* to multiple antibacterial agents, including β-lactams, macrolides, tetracyclines, and co-trimoxazole, has emerged worldwide in the 1980s and 1990s (1–3) and has emphasized the need for new therapeutic alternatives, such as newer fluoroquinolones. Older fluoroquinolones, such as ciprofloxacin and ofloxacin, have been widely used in the last 2 decades, but their activity against gram-positive pathogens is limited. Newer fluoroquinolones, such as levofloxacin, gatifloxacin, moxifloxacin, and gemifloxacin, have enhanced activity against most respiratory pathogens, and some are being more widely used to treat respiratory tract infections. Therefore, the emergence of fluoroquinolone-resistant *S. pneumoniae* strains, although worldwide prevalence is low, is a concern to clinicians who manage respiratory tract infections. A global surveillance study from 1998 to 2000 included 8,882 pneumococci isolated from blood and sputum samples that were collected from centers in 26 countries. The study showed a 1.1% prevalence of ofloxacin-resistant strains, although in some places higher values of nonsusceptible ofloxacin strains were observed: 10.1% in Israel, 14.1% in Japan, and 22.3% in Hong Kong (1). Ciprofloxacin-resistant strains isolated from patients with community-acquired respiratory tract infections have been reported in Spain (3.0% [4] and 7.1% [5]), Canada (1.7% [6]), and the United States (1.4% [7]).

Fluoroquinolone resistance is higher among related viridans group streptococci (VGS) isolated from blood (8). Increased fluoroquinolone use correlates with an increase in the prevalence of ciprofloxacin resistance (4,6). Prior fluoroquinolone administration is a risk factor for resistant strain selection, as observed for infections caused by *S. pneumoniae* (9–12) and VGS (13). A study from our group

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showed ciprofloxacin-resistant pneumococci emerging in a patient who had received long-term ciprofloxacin therapy to treat persistent bronchiectasis with *Pseudomonas aeruginosa* infection (14).

Bacterial resistance to quinolones occurs mainly by alteration of their intracellular drug targets, the DNA topoisomerase IV ($ParC_2ParE_2$) and DNA gyrase ($GyrA_2GyrB_2$) enzymes. The pneumococcal *parC* and *parE* genes are homologous to *gyrA* and *gyrB*, respectively (15,16). Genetic and biochemical studies have shown that fluoroquinolones target primarily topoisomerase IV and secondarily DNA gyrase in *S. pneumoniae* (16–19). Resistance mutations are localized in the quinolone resistance-determining regions (QRDRs) of ParC, ParE, and GyrA. Low-level ciprofloxacin-resistant strains had mutations altering the QRDR of one of the two subunits of topoisomerase IV: S79 or D83 of ParC (16,17,20) or D435 of ParE (21). High-level ciprofloxacin-resistant strains had changes affecting both QRDRs of ParC and GyrA (S81, E85) (16,17) or ParE and GyrA (21). Genetic transformation experiments showed that single *parC* mutations confer low-level ciprofloxacin resistance, and that once the cells have acquired this phenotype, transforming to a higher level of resistance was possible by using DNA containing the *gyrA* QRDR from the high-level ciprofloxacin-resistant strains (16,17). The description of recombinant strains with a mosaic structure in their DNA topoisomerase genes (22–24) has established that fluoroquinolone resistance in *S. pneumoniae* can be acquired by horizontal gene transfer as well as by point mutation. VGS, which share the same mechanisms of resistance (13), are donors in the horizontal transfer to pneumococci (25) and act as a reservoir of fluoroquinolone resistance. To investigate the epidemiology of fluoroquinolone-resistant pneumococci, ascertain the possible dissemination of international clones, and determine the incidence of resistant strains originated by interspecific horizontal transfer, we characterized all ciprofloxacin-resistant *S. pneumoniae* strains sent to Spanish Reference Laboratory during 2002.

Materials and Methods

Bacterial Strains, Serotyping, and Susceptibility Tests

We studied 2,882 *S. pneumoniae* strains submitted from 78 hospitals nationwide to the Spanish Reference Laboratory during 2002. Of the submitted strains, 1,904 (66.1%) were isolated from adults and 978 (39.1%) from children. The origin of isolates was as follows: 1,453 (50.4%) from blood or other sterile sites, 691 (24.0%) from respiratory tract, 231 (8.0%) from eye swabs, 205 (7.1%) from ear swabs, and 302 (10.5%) from other sites. Strains were confirmed as *S. pneumoniae* by standard methods, and serotypes were determined by a quellung reaction. Susceptibility tests, performed initially by the agar-dilution method, selected 85 strains (one isolate per patient) with ciprofloxacin MIC ≥ 4 μg/mL. The susceptibility of these 85 strains was repeated twice by microdilution (Sensititre commercial plates) according to the National Committee for Clinical Laboratory Standards (NCCLS) methods (26). *S. pneumoniae* ATCC 49619 and strain R6 were used for quality control. Fluoroquinolone efflux was determined (27).

Genetic Transformation

S. pneumoniae strain R6 was grown in a casein hydrolysate-based medium (AGCH) with 0.2% sucrose and used as recipient in transformation experiments (20). Cultures containing 4 x 10^6 CFU/mL were treated with DNA at 0.15 μg/mL for 40 min at 30°C, then at 37°C for 90 min before plating on media plates containing 2.5 μg/mL of ciprofloxacin. Colonies were counted after 24 h growth at 37°C in a 5% CO_2 atmosphere in AGCH medium with 1% agar.

Southern Blot Analysis

Probes for *parC* and *parE* were amplified by polymerase chain reaction (PCR) of the R6 strain with oligonucleotides parCUP and parCDOWN, parEUP and parEDOWN, respectively (25). The *ant* probe was obtained by amplifying strain 3870 DNA (22) with oligonucleotides antUP and antDOWN (25). All probes were labeled with the Phototope-Star Detection Kit (New England Biolabs, Beverly, MA). Southern blot and hybridization followed the manufacturer's instructions.

Pulsed-field Gel Electrophoresis (PFGE)

Genomic DNA embedded in agarose plugs was restricted with *Sma*I, and fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA) (28). PFGE patterns were compared to 14 representative international pneumococcal clones of the Pneumococcal Molecular Epidemiology Network (28). Strains with patterns varying by three or fewer bands were considered to represent the same PFGE type (29).

PCR Amplifications and DNA Sequence Determination

Oligonucleotides gyrA44 and gyrA170 (13) were used to amplify and sequence the *gyrA* QRDRs from chromosomal DNA. Amplifications were performed with 0.5 U of *Thermus thermophilus* thermostable DNA polymerase (Biotools, Madrid, Spain), 0.1 μg of chromosomal DNA, 1 μmol/L (each) of the synthetic oligonucleotide primers, and 0.2 mmol/L of each deoxynucleoside triphosphate (dNTP) in the buffer recommended by the manufacturers. Amplification was achieved with an initial cycle of 1 min denaturation at 94°C; 25 cycles of 30 s at 94°C, 45 s at

55°C, and 90 s polymerase extension step at 72°C; and a final 3-min 72°C extension step, followed by slow cooling to 4°C. Fragments including ParE residues 398–647, the intergenic *parE–parC* region, and ParC residues 1–152 were amplified from genomic DNA with oligonucleotides parE398 (13) and parC152 (16) in PCR reactions performed as previously described, except that 30 cycles of amplification with an extension step of 3 min were applied. All strains, except three, CipR-73, CipR-74, and CipR-75, which yielded fragments of 2.4, 3.0, and 2.9 kb, respectively, yielded 1.6-kb fragments. Those PCR fragments that contained both *parE* and *parC* QRDRs were purified by using MicroSpin S400 HR columns (Amersham Pharmacia Biotech, Pistcatway, NJ) and sequenced on both strands with oligonucleotides parE398, parE483 (13), parC50, and parC152 (16) with an Applied Biosystems Prism 377 DNA sequencer, according to protocols provided by the manufacturer.

Statistical Analysis

The Fisher exact test (χ^2 test) was used when appropriate. Comparisons were considered significant at $p < 0.05$.

Results

Among 2,882 strains, 75 (2.6%) had ciprofloxacin MIC ≥4 μg/mL by microdilution and were considered ciprofloxacin-resistant. Ten strains with initial ciprofloxacin MIC 4 μg/mL by agar-dilution showed MIC 1–2 μg/mL by microdilution. Among the 75 ciprofloxacin-resistant strains, 67% were isolated from sputum, whereas 19% were from blood, 13% from lower respiratory tract samples, and 1.3% from pus. Although ciprofloxacin-resistant strains belonged to 17 different serotypes, 6 serotypes accounted for 55 (73.3%) of the strains: 14 (14 strains), 23F (11 strains), 19F (11 strains), 6B (8 strains), 3 (6 strains), and 15A (5 strains). These serotypes were also the most frequent among the 2,807 ciprofloxacin-susceptible strains. No ciprofloxacin-resistance was found among isolates from pediatric patients <15 years, whereas the prevalence of resistance was 75 (3.9%) of 1,904 isolates from adult patients (≥15 years), and 53 (7.2%) of 738 isolates from patients ≥65 years ($p < 0.001$). The frequencies of ciprofloxacin resistance were higher among noninvasive pneumococci than among invasive strains (61 [4.3%] of 1,429 vs. 14 [1.0%] of 1,453, $p < 0.001$).

An association between penicillin and ciprofloxacin resistance was observed, with ciprofloxacin-resistant strains distributed as follows: 17 (1.0%) of 1,658 penicillin-susceptible, 39 (4.1%) of 961 intermediate-resistant, and 19 (7.2%) of 263 resistant isolates ($p < 0.001$). Ciprofloxacin-resistant pneumococci were also more represented among macrolide-resistant isolates: 22 (1.2%) ciprofloxacin-resistant strains among 1,833 erythromycin-susceptible and 53 (5.1%) ciprofloxacin-resistant strains among 1,049 erythromycin-resistant ($p < 0.001$). Antimicrobial-drug resistance among the 2,807 ciprofloxacin-susceptible pneumococci was lower than those of ciprofloxacin-resistant strains (penicillin 41.4% vs. 73.3%, erythromycin 35.5% vs. 70.7%, tetracycline 33.6% vs. 69.3%, and chloramphenicol 15.4% vs. 44.0%, $p < 0.001$). Fifty-five (73.3%) of ciprofloxacin-resistant strains showed multidrug resistance (resistant to more than three chemically unrelated drugs), among them, 26 strains were resistant to six antimicrobial agents (penicillin, tetracycline, chloramphenicol, erythromycin, clindamycin, and co-trimoxazole).

Fourteen (18.7%) ciprofloxacin-resistant (MIC 4–8 μg/mL) strains were classified as low-level ciprofloxacin-resistant, and 61 (81.3%) strains were classified as high-level ciprofloxacin-resistant (MIC 16–128 μg/mL). The comparative in vitro activity of five fluoroquinolones (MIC_{90}) against the ciprofloxacin-resistant strains was the following: gemifloxacin (0.5 μg/mL) > moxifloxacin (4 μg/mL) > gatifloxacin (8 μg/mL) > levofloxacin (32 μg/mL) > ciprofloxacin (64 μg/mL) (Table 1). Gemifloxacin was the most active fluoroquinolone tested by using the NCCLS breakpoint (26), 49.3% of ciprofloxacin-resistant strains were nonsusceptible to this antimicrobial drug. Among the 14 low-level ciprofloxacin-resistant isolates, 12 were susceptible to levofloxacin, 13 to gatifloxacin, and all were susceptible to moxifloxacin and gemifloxacin according to NCCLS criteria (26). However, all but one of these low-level ciprofloxacin-resistant strains had first-step *parC* mutations that would favor the appearance of high-level resistant strains. Among the 61 high-level ciprofloxacin-resistant strains, all showed cross-resistance to levofloxacin and gatifloxacin and all but one to moxifloxacin. Twenty four (39.3%) of 61 high-level ciprofloxacin-resistant strains had gemifloxacin MIC 0.12 μg/mL, which could be considered susceptible according to NCCLS (26), although they showed double mutations (*parC* or *parE* and *gyrA*). One of 75 ciprofloxacin-resistant strains, strain CipR-71, with MIC 8 μg/mL, had an efflux mechanism as the single cause of resistance (see below).

The *parC*, *parE*, and *gyrA* QRDRs of 85 strains (75 ciprofloxacin-resistant strains and 10 strains with ciprofloxacin MIC 1–2 μg/mL) were characterized. Most resistant strains (70 of 75) showed low nucleotide sequence variations (≤1%), but five strains had high variations (>4%) in at least one of their QRDRs (Figure). These strains would have a mosaic structure in those genes showing such a QRDR sequence variation (25): two strains in *parC*, one in *parC* + *parE*, and two in *parC* + *parE* + *gyrA*. Mosaic *parC* genes had the N91D change, and mosaic *gyrA* genes had the S114G GyrA change (Figure) that is also present in other ciprofloxacin-resistant *S. pneumoniae*

Table 1. In vitro activity of 13 antimicrobial drugs against 75 ciprofloxacin-resistant *Streptococcus pneumoniae* isolates[a]

Drug	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC range (μg/mL)	Susceptible breakpoints	%S	%I	%R	%I+R
Penicillin	1	2	0.03–8	≤ 0.06	26.7	48.0	25.3	73.3
Amoxicillin	1	4	0.06–16	≤ 2	80.0	12.0	8.0	20.0[b]
Cefotaxime	0.5	1	0.03–8	≤ 1	92.1	5.3	2.6	7.9[b]
				≤ 0.5	56.1	36.0	7.9	43.9[c]
Erythromycin	128	128	0.06–>256	≤ 0.25	29.3	0.0	70.7	70.7
Clindamycin	128	128	0.06–>256	≤ 0.25	37.3	0.0	62.7	62.7
Tetracycline	32	64	0.12–64	≤ 2	30.7	2.7	66.6	69.3
Chloramphenicol	2	16	2–32	≤ 4	56.0		44.0	44.0
Cotrimoxazole	≥4/76	≥4/76	0.5/9.5–≥4/76	≤ 0.5/9.5	32.0	4.0	64.0	68.0
Ciprofloxacin[d]	32	64	4–128	NA	NA	NA	NA	
Levofloxacin	16	32	1–64	≤ 2	16.0	2.7	81.3	84.0
Gatifloxacin	4	8	0.5–16	≤ 1	17.3	2.7	80.0	82.7
Moxifloxacin	2	4	0.12–8	≤ 1	20.0	37.3	42.7	80.0
Gemifloxacin	0.12	0.5	0.06–2	≤ 0.12	50.7	28.0	21.3	49.3

[a]S, susceptible; I, intermediate; and R, resistant, according to National Committee for Clinical Laboratory Standards (NCCLS) 2004 interpretative criteria; NA, not available.
[b]NCCLS 2004 nonmeningitis criteria.
[c]NCCLS 2004 meningitis criteria.
[d]Ciprofloxacin resistance defined as ≥4 μg/mL by Chen et al. (4).

strains with mosaic genes and in both ciprofloxacin-susceptible and -resistant VGS (25), which indicates their recombinational origin. As expected (25), strains with mosaic *parE* + *parC* genes had also an *ant*-like gene in the intergenic *parE–parC* region (Figure).

All low-level ciprofloxacin-resistant strains had mutations producing amino acid changes in ParC, whereas 92% of high-level resistant strains had double (ParC + GyrA or ParE + GyrA) and 8% had triple mutations (two changes in ParC + GyrA, ParC + ParE + GyrA, ParC + two changes in GyrA). Classical mutations known to be involved in fluoroquinolone resistance were found in all but two resistant strains with mosaic *parC* genes (Table 2): D78A (strain CipR-71) and S79R (strain CipR-75). Genetic transformation experiments showed that the S79R change was involved in ciprofloxacin resistance and that the D78A change was not. CipR-71 and CipR-75 chromosomal DNA transformed the susceptible R6 strain to resistance with high efficiency (>1 x 10^4 transformants per microgram of DNA). The analysis of two transformants of each experiment showed that, while transformants obtained with CipR-71 DNA (ParC D78A) did not carry *parC* mutations, those obtained with CipR-75 (ParC S79R) carried the same nucleotide changes in *parC* as the donor strain.

When PCR products carrying ParE residues 398–647, the intergenic *parE–parC* region, and ParC residues 1–152 (including ParE and ParC QRDRs) were used as DNA donors, only those obtained from strain CipR-75 were able to transform R6 to ciprofloxacin resistance. The two transformants selected carried the ParC S79R change, and one of them also had the N91D change. These results showed that the S79R change is involved in the resistance phenotype of strain CipR-75 and that the D78A change of strain CipR-71 is not involved in resistance. Determining MIC to ciprofloxacin, norfloxacin, ethidium bromide, and acriflavine in the presence or absence of reserpine showed greater than fourfold MIC decreases in the presence of reserpine, both in the CipR-71 strain and in the two transformants obtained when chromosomal DNA was used as donor, which indicates the existence of an efflux mechanism (data not shown).

Although 36 different PFGE patterns were observed in the 75 ciprofloxacin-resistant strains, 48 strains can be grouped into nine PFGE patterns (Table 3): 11 strains belonged to Spain^{23F}-1 clone, 8 strains to Spain^{14}-5 clone, 6 strains to Spain^{9V}-3 clone, 5 strains to Spain^{6B}-2 clone, 5 strains to Sweden^{15A}-25 clone, 4 strains to clone C of serotype 19F, 4 strains to clone D of serotype 19F, 3 strains to clone A of serotype 3 related to multilocus sequence type (MLST) 260 (30), and 2 strains to clone B of serotype 3 related to MLST 180 (30). Among 14 blood isolates, 12 different PFGE types were observed. Only three of these strains (CipR-1, -2, and -15) shared an identical PFGE (clone A of serotype 3), although they had different *parE* polymorphisms and different resistance patterns (Table 2).

One of the strains with a mosaic *parC* gene belonged to the Spain^{6B}-2 clone, and one strain with mosaic *parC* and *parE* genes belonged to the Spain^{23F}-1 clone. Capsular switching was observed in three strains of serotype 14 with the Spain^{9V}-3 clone, one strain of serotype 19F with Spain^{9V}-3 clone, and two strains of serotype 19A with Spain^{23F}-1 clone. In general, strains that shared the same PFGE pattern shared identical polymorphisms on their DNA topoisomerase QRDRs with respect to the sequence of the R6 strain (Table 3). For instance, all strains of the Spain^{9V}-3 clone had identical polymorphisms, the same found in the ATCC 700671 strain representative of this clone (14). All but one of the strains belonging to the

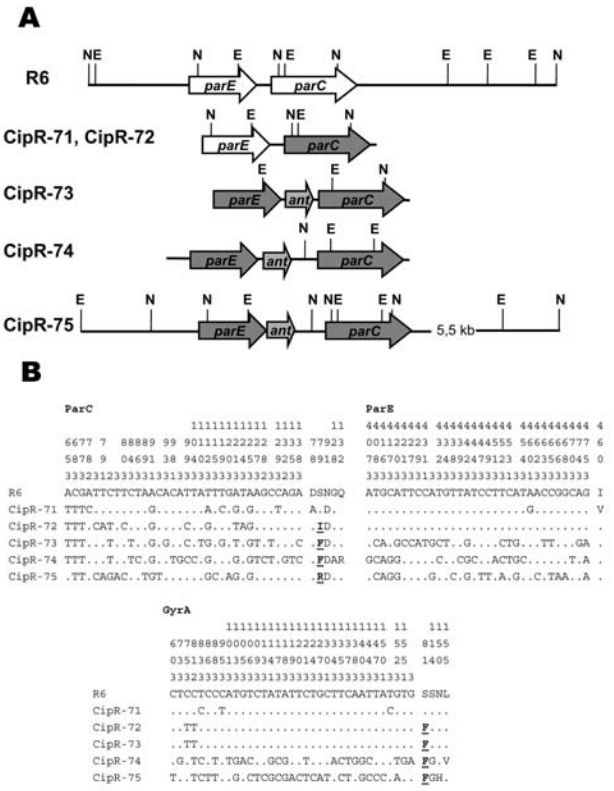

Figure. Genetic organization of the *parE–parC* region of *Streptococcus pneumoniae* mosaic strains and nucleotide sequence variations in the ParC, ParE, and GyrA quinolone resistance-determining regions. A) Structure as deduced from Southern blot experiments and nucleotide sequence analyses. E, *Eco*RV; N, *Nco*I. The *parE* and *parC* genes with a mosaic structure are shown with darker gray arrows. B) Nucleotides present at each polymorphic site are shown for strain R6, but only sites that differ are shown for the other strains. Amino acid changes involved in fluoroquinolone resistance are shown in **boldface** and underlined. Codon numbers are indicated in vertical format above the sequences. Positions 1, 2, and 3 in the fourth row refer to the first, second, and third nucleotides in the codon.

Spain^{23F}-1 clone had two polymorphisms in ParC (K137N and a change in codon G128: GGT instead of GGC), two polymorphisms in ParE (I460V and a change in codon I476: ATT instead of ATC), and a change in codon Y75 of GyrA (TAT instead of TAC). Identical polymorphisms were found in the ATCC 700669 strain representative of the Spain^{23F}-1 clone (data not shown); the only exception was the Spain^{23F}-1 strain CipR-73 that had *parC* and *parE* mosaic genes (Table 3). Other exceptions were three strains of the Spain^{14}-5 clone (CipR-38, CipR-39, and CipR-40) that had an additional GyrA polymorphism (V88I), which were isolated from three patients temporally related in the same hospital, and two other strains, CipR-1 of clone A of serotype 3 and CipR-57 of clone D of serotype 19F that showed different polymorphisms in ParE (Table 3).

Discussion

The worldwide prevalence of fluoroquinolone resistance in *S. pneumoniae* is low, although it varies over time, geographic region, age group, and origin of isolates (1,4,6,31). The overall incidence of ciprofloxacin-resistant *S. pneumoniae* strains in this study was 2.6%, lower than that reported in previous studies (3%–7%) (4,5) conducted in Spain in which noninvasive isolates were predominant. In agreement with those previous studies, noninvasive isolates in this study displayed ciprofloxacin resistance as high as 4.3%. A higher prevalence (7.2%) was seen in patients ≥65 years, which also agreed with previous reports (4,6), and which possibly reflects increased fluoroquinolone use in this group of patients. The prevalence of ciprofloxacin resistance seen in this study (2.6%) is similar to the 2.5% found among unselected isolates of Bellvitge Hospital (Barcelona) in 2002 (J. Liñares, pers. comm.), but it is lower than the 6.3% found in Donostia Hospital (San Sebastian) (E. Pérez-Trallero, pers. comm.).

A significant relation between resistance to penicillin or macrolides and resistance to ciprofloxacin was observed (Table 1), in agreement with previous reports (4,6). Penicillin- and multidrug-resistant pneumococci have been common in Spain since the 1980s; these strains primarily belong to serogroup 19 and to four multiresistant epidemic clones: Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, and Spain^{14}-5 (30,32,33). The emergence of fluoroquinolone resistance among strains of these clones has been described, (33–36) and in our study, resistance occurred mainly among those four widely disseminated clones (Table 3). Since neither temporal nor geographic relationships were found among most patients infected by these four clones, this finding could reflect the high prevalence of these clones in the Spanish population, as suggested previously (36), rather than the spreading of resistant clones. Exceptions were five strains of the Spain^{14}-5 clone that could be grouped in two temporal clusters. One cluster accounted for two strains (CipR-66 and -67) collected in the same laboratory, with identical polymorphisms and triple mutations in QRDRs. The second cluster accounted for three identical strains of the Spain^{14}-5 clone (CipR-38, -39 and -40), which were recovered from three patients at a laboratory from San Sebastian in a 29-day period. These strains had an additional GyrA polymorphism (V88I) not present in other Spain^{14}-5 clone strains and may represent a variant of this clone (33). Nevertheless, the epidemiologic features of these international clones (2,37,38) suggest that dissemination of ciprofloxacin resistance through these isolates is a plausible scenario and may predict a rapid increase of resistance in *S. pneumoniae* in countries with an increasing use of fluoroquinolones. Also plausible is that recombinant ciprofloxacin-resistant isolates with

Table 2. Fluoroquinolone MIC of 85 strains and amino acid changes in their DNA topoisomerase genes[a]

No. strains	Amino acid substitution			MIC (μg/mL)				
	ParC	Par E	GyrA	CIP	LVX	GAT	MXF	GEMI
10	None	None	None	1–2	1–2	0.12–0.5	0.12–0.25	0.015–0.03
1	None	None	None	8	2	0.5	0.12	0.06
8	S79F	None	None	4–8	2–4	0.5–2	0.12–0.5	0.03–0.06
3	S79Y	None	None	4–8	2–4	0.5	0.25	0.03–0.06
1	D83G	None	None	4	1	0.5	0.25	0.03
1	D83Y	None	None	4	1	0.5	0.25	0.03
1	S79A	None	S81F	16	8	4	2	0.12
21	S79F	None	S81F	32–128	8–32	4–8	2–4	0.12–1
9	S79F	None	S81Y	32–64	8–16	4–8	1–4	0.12–0.25
6	S79F	None	E85K	32–64	16–64	4–16	2–8	0.25–2
6	S79Y	None	S81F	32–64	8–64	4–8	2–4	0.12–0.25
1	S79Y	None	S81Y	32	16	8	4	0.12
2	D83N	None	S81F	32–64	8–16	2–4	2	0.12–0.25
1	D83N	None	S81F	32	16	8	4	0.5
3	None	D435N	S81F	16–32	8–16	4–8	2–4	0.12–0.25
1	None	D435N	S81Y	16	16	4	2	0.12
1	S79Y, D83N	None	E85K	64	32	8	4	1
3	S79F	D435N	E85K	64	64	8–16	8	0.5
1	S79Y	D435N	E85K	64	32	8	4	0.5
1	S79F	None	S81F, E85A	64	64	16	8	1
1	S79I	None	S81F	32	16	4	2	0.12
1	S79F	None	S81F	32	16	4	4	0.5
1	S79F	None	S81F	32	8	4	2	0.25
1	S79R	None	S81F	32	16	8	4	0.5

[a]Only changes involved in resistance are shown, and double underlining indicates that the residue is located in a gene with a mosaic structure. Additional amino acid changes, not involved in resistance, were: ParC D78A (the CipR-71 strain with no mutations that has a mosaic *parC* gene), R36C (3 strains), ParC K137N (27 strains), ParC N91D (the three strains with mosaic *parC* genes), ParE I460V (40 strains), ParE453S (1 strain), GyrA V88I (3 strains), GyrA S114G (the two strains with mosaic *gyrA* genes), and GyrA N150H (one of the two strains with a mosaic *gyrA* gene). CIP, ciprofloxacin; LVX, levofloxacin; GAT, gatifloxacin; MXF, moxifloxacin; GEMI, gemifloxacin.

mosaic DNA topoisomerase genes belonging to the Spain^{23F}-1 and Spain^{6B}-2 international clones (Table 3) have disseminated and that these isolates have acquired resistance mutations by horizontal transfer from VGS. Although a low prevalence (5 [6.7%] of 75) of *S. pneumoniae* strains with mosaic *parC* genes among the ciprofloxacin-resistant strains was observed, the existence of these kind of strains (Figure) is an indication that recombination has occurred between resistant VGS and *S. pneumoniae*, including the prevalent pneumococcal clones. In addition, five resistant strains of the Sweden^{15A}-25 clone, another multiresistant clone previously reported as susceptible (28), were found (Table 3).

Although newer fluoroquinolones have enhanced in vitro activity against *S. pneumoniae* and are less likely to select for resistant isolates, their use to treat respiratory tract infections merits special attention. More than 20 reports of levofloxacin treatment failure have been concurrent with the development of resistance during or after therapy. In some cases, strains susceptible to levofloxacin but with a first-step *parC* mutation, as a consequence of previous quinolone use, were present before therapy was initiated. Identifying these strains will help avoid therapeutic failures. Since fluoroquinolone resistance is more frequent in the elderly with chronic respiratory diseases who have had long-term quinolone therapy (4,6,10–12,14), recent use of these drugs should contraindicate further fluoroquinolone treatment.

The use of NCCLS susceptibility breakpoints for newer fluoroquinolones underestimates a high proportion of low-level ciprofloxacin-resistant strains with first-step mutations whose detection should be improved by decreasing those breakpoints as previously suggested (11,39). In the present study, which followed NCCLS criteria (26), 12 (85%) of 14 low-level ciprofloxacin-resistant strains with a single *parC* mutation were susceptible to levofloxacin (MIC 1–2 μg/mL). These data agree with those of other authors who found *parC* mutations in 59% of the strains with levofloxacin MIC 2 μg/mL (39). Using a ciprofloxacin resistance breakpoint MIC ≥4 μg/mL, as was used by Chen et al. (6), would improve detection of these mutant strains. In fact, in our study, no strains with first-step mutations and ciprofloxacin MIC 2 μg/mL were detected, although other authors have found these mutations in up to 29% of this type of strain (39). Although gemifloxacin was the most active quinolone studied, and 39.3% of high-level ciprofloxacin resistance with double mutations were gemifloxacin-susceptible with the NCCLS

Table 3. Summary of phenotypic characteristics and changes in the QRDR among the most prevalent pulsed-field gel electrophoresis patterns of ciprofloxacin-resistant strains[a]

PFGE	Strain	Serotype	Resistance pattern	aa and nt changes in QRDR		
				ParC	ParE	GyrA
Referent	R6	NT	S	None	None	None
Spain^{23F}-1	CipR-8	23F	PEClTCSxTCp	G128 (GGT), K137N, **S79F**	I460V, I476 (ATT)	Y75 (TAT)
	CipR-5	19A[b]	PTCSxTCp	G128 (GGT), K137N, **S79F**	I460V, I476 (ATT)	Y75 (TAT)
	CipR-9	23F	PEClTCSxTCp	G128 (GGT), K137N, **S79Y**	I460V, I476 (ATT)	Y75 (TAT)
	CipR-12	23F	PTCCp	G128 (GGT), K137N, **D83G**	I460V, I476 (ATT)	Y75 (TAT)
	CipR-30, -31, -32, -33	23F	PEClTCSxTCp	G128 (GGT), K137N, **S79F**	I460V, I476 (ATT)	Y75 (TAT), **S81F**
	CipR-48, -49	23F	PEClTCSxTCp	G128 (GGT), K137N, **S79F**	I460V, I476 (ATT)	Y75 (TAT), **E85K**
	CipR-73	19A[b]	EClTSxTCp	**S79F**	None	Y75 (TAT), **S81F**
Spain^{14}-5	CipR-24	14	PEClTCSxTCp	**S79F**	*None*	Y75 (TAT), **S81F**
	CipR-41	14	PEClTCSxTCp	**S79F**	None	Y75 (TAT), **S81Y**
	CipR-55	14	PEClTCSxTCp	**S79Y**	None	Y75 (TAT), **S81F**
	CipR-66, -67	14	PEClTCSxTCp	**S79F**	**D435N**	Y75 (TAT), **E85K**
	CipR-38, -39, -40	14	PEClTCSxTCp	**S79F**	None	Y75 (TAT), V88I, **S81Y**
Spain^{9V}-3	CipR-10	9V	PSxTCp	K137N, **S79Y**	I460V	Y75 (TAT)
	CipR-14	14[b]	PESxTCp	K137N, **S79A**	I460V	Y75 (TAT), **S81F**
	CipR-51	14[b]	PSxTCp	K137N, **S79Y**	I460V	Y75 (TAT), **S81F**
	CipR-20	9V	PSxTCp	K137N, **S79F**	I460V	Y75 (TAT), **S81F**
	CipR-28	19F[b]	PSxTCp	K137N, **S79F**	I460V	Y75 (TAT), **S81F**
	CipR-58	14[b]	PSxTCp	K137N, **D83N**	I460V	Y75 (TAT), **S81F**
Spain^{6B}-2	CipR-3	6B	PEClTSxTCp	**S79F**	None	Y75 (TAT)
	CipR-17, -18, -19	6B	PEClTCSxTCp	**S79F**	None	Y75 (TAT), **S81F**
	CipR-72	6B	PEClTSxTCp	**S79F**	None	Y75 (TAT), **S81F**
Sweden^{15A}-25	CipR-4	15A	PEClTCp	G77 (GGA), **S79F**	I460V	Y75 (TAT)
	CipR-64	15A	PEClTCp	G77 (GGA)	I460V, **D435N**	Y75 (TAT), **S81Y**
	CipR-60	15A	PEClTCp	G77 (GGA), **D83Y**	I460V	Y75 (TAT), **E85K**
	CipR-45, -50	15A	PEClTCp	G77 (GGA), **S79F**	I460V	Y75 (TAT), **E85K**
C	CipR-59	19F	PTCSxTCp	**D83N**	None	Y75 (TAT), **S81F**
	CipR-43, -44	19F	PTCSxTCp	**S79F**	None	Y75 (TAT), **S81Y**
	CipR-65	19F	PEClTCSxTCp	**S79Y**, **D83N**	None	Y75 (TAT), **E85K**
D	CipR-57	19F	PEClTCp	G77 (GGA), **S79Y**	I460V, P454S	Y75 (TAT), **S81Y**
	CipR-29	19F	PEClTCCp	G77 (GGA), **S79F**	I460V	Y75 (TAT), **S81F**
	CipR-46, -47	19F	PEClTCp	G77 (GGA), **S79F**	I460V	Y75 (TAT), **E85K**
A	CipR-1	3	Cp	R95C, **S79F**	I460V	Y75 (TAT), H104 (CAC)
	CipR-2	3	Cp	R95C, **S79F**	None	Y75 (TAT), H104 (CAC)
	CipR-15	3	TCCp	R95C, **S79F**	None	Y75 (TAT), H104 (CAC), **S81F**
B	CipR-61	3	Cp	None	I460V, **D435N**	Y75 (TAT), **S81F**
	CipR-52	3	ECp	**S79Y**	I460V	Y75 (TAT), **S81F**

[a]QRDR, quinolone-resistance determining regions; PFGE, pulse-field gel electrophoresis *Sma*I patterns; aa, amino acid; nt, nucleotide; S, susceptible to all antibiotics tested; P, resistant to penicillin (MIC 0.12–4 μg/mL); T, resistant to tetracycline (MIC ≥4 μg/mL); C, resistant to chloramphenicol (MIC ≥8 μg/mL); E, resistant to erythromycin (MIC ≥0.5 μg/mL); Cl, resistant to clindamycin (MIC ≥0.5 μg/mL); SxT, resistant to trimethropin-sulfamethoxazole (MIC ≥4/76 μg/mL); Cp, resistant to ciprofloxacin (MIC ≥4 μg/mL). Residue changes involved in fluoroquinolone resistance are showed in **boldface**, and double underlining indicates that the residue is located in a gene with a mosaic structure. A, PFGE type related to serotype 3 with MLST 260; B, PFGE type related to serotype 3 with MLST 180; NT, non typeable.
[b]Capsular switching.

breakpoint, a patient infected by such strains should not be treated with any quinolone. To avoid treatment failures, using microbiologic breakpoints for quinolone susceptibility would be more prudent than using clinical breakpoints. If fluoroquinolones are widely and indiscriminately used, resistance to fluoroquinolones in *S. pneumoniae* may become a problem in the near future.

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Close Call

All my life my father refused to talk about
his boyhood in Norway. "No," he'd say when
I cajoled him for details. "I'm an American now."

The only thing he'd ever talk about was how he'd
ended up in Minneapolis at Augsburg Seminary,
the story of his "close call," as he referred to it.

He was the only one of his three brothers and sister
who emigrated. "He broke our mother's heart," my aunt
told me when I visited her in Norway many years later.

She gave me the picture she'd taken the day he left, the
day after Christmas, 1920. He's impossibly young, already
wearing his life-long uniform - black suit, vest, white shirt, tie,

ready to go off to America, even if his mother's
heart is breaking, because he had to fulfill a promise
he made when he got the Spanish Flu, summer of 1918.
"Twenty two million people died," he was fond of telling me,
"twice as many as died in World War I, but I didn't die.
When I was choking and close to death, my mother

called the village doctor who performed a tracheotomy
right on our kitchen table and I promised then I'd serve
God forever if He wouldn't let me die. It was a close call."

Close call, I say, echoing my father, now dead these 20 years.
How close he came to being one of the 22 million, how he
almost didn't make it to America, almost didn't spend a

summer in Duluth, preaching at the Norwegian Seaman's
Mission, almost didn't meet my mother whose youth group
was serving coffee and cake after the service, almost didn't

marry her, almost didn't make love with her that warm June
evening of 1927, the night I was conceived, in the white frame
parsonage in Bagley, Minnesota. Close call. Close call.

Phebe Hanson (b. 1928)
From Why Still Dance, Nodin Press, 2003.
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Sulfa Use, Dihydropteroate Synthase Mutations, and *Pneumocystis jirovecii* Pneumonia

Cheryl R. Stein,* Charles Poole,* Powel Kazanjian,† and Steven R. Meshnick*

A systematic review was conducted to examine the associations in *Pneumocystis jirovecii* pneumonia (PCP) patients between dihydropteroate synthase (DHPS) mutations and sulfa or sulfone (sulfa) prophylaxis and between DHPS mutations and sulfa treatment outcome. Selection criteria included study populations composed entirely of PCP patients and mutation or treatment outcome results for all patients, regardless of exposure status. Based on 13 studies, the risk of developing DHPS mutations is higher for PCP patients receiving sulfa prophylaxis than for PCP patients not receiving sulfa prophylaxis ($p < 0.001$). Results are too heterogeneous ($p < 0.001$) to warrant a single summary effect estimate. Estimated effects are weaker after 1996 and stronger in studies that included multiple isolates per patient. Five studies examined treatment outcome. The effect of DHPS mutations on treatment outcome has not been well studied, and the few studies that have been conducted are inconsistent even as to the presence or absence of an association.

Pneumocystis jirovecii causes pneumonia in immunocompromised persons, especially those with AIDS, worldwide (1). In industrialized countries, while the incidence of *Pneumocystis jirovecii* pneumonia (PCP) has declined substantially since highly active antiretroviral therapy (HAART) was introduced in 1996 (2), PCP remains the leading serious opportunistic infection (3–5). Not all patients treated with HAART have CD4-cell count boosts above the range at which PCP occurs (6–9). In developing countries, where only 7% of HIV/AIDS patients who need therapy have access to HAART (10), the incidence of PCP is increasing.

Prophylaxis against PCP has been standard practice in industrialized countries for >20 years. Trimethoprim-sulfamethoxazole (TMP-SMX) is the first-line drug choice for both prophylaxis and therapy. TMP-SMX acts in animals as sulfa monotherapy against the enzyme dihydropteroate synthase (DHPS) (11,12). Dapsone, a sulfone drug also targeting DHPS, is frequently used as a second-line agent for prophylaxis and treatment of PCP.

Failure of sulfa or sulfone (sulfa) prophylaxis against PCP has been reported in up to one fourth of patients (13,14). To assess the role of drug resistance in these failures, investigators examined whether DHPS mutations are more frequent among patients with or without prior exposure to sulfa agents, and whether infections in patients with or without DHPS mutations are more likely to be unresponsive to a sulfa drug. These studies are hampered by scientists' inability to culture *P. jirovecii*, which prevents direct confirmation of resistance through standard drug-susceptibility testing. Instead, researchers use polymerase chain reaction to detect *P. jirovecii* DHPS mutations that cause sulfa resistance in other microorganisms. DHPS mutations in *P. jirovecii* may also increase the incidence of treatment failure. A systematic review can determine whether available studies give overall evidence of an association, assess the possibility of publication bias, examine results across studies for consistency, and investigate study and patient characteristics for possible influence on study results.

Methods

Literature Search

MEDLINE (National Library of Medicine, Bethesda, MD) was searched with the keywords "*Pneumocystis*," "*Pneumocystis carinii*," and "drug resistance" (last searched January 2004). ISI Web of Science (Institute for Scientific Information, Philadelphia, PA) was searched with the keywords "pneumocystis pneumonia," "resistance," and "genes" (last searched January 2004). The bibliographies of relevant articles were surveyed for

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additional studies. One author (S.R.M.) contacted 42 scientists through an informal PCP email forum to request unpublished results and conference abstracts on associations between sulfa prophylaxis and *Pneumocystis* mutations and *Pneumocystis* mutations and sulfa treatment outcome.

Information Extraction

Inclusion requirements were the following: study populations composed entirely of PCP patients; mutation results for all patients, regardless of sulfa prophylaxis exposure; and treatment outcome results for all patients, regardless of mutation status. Studies reporting the outcome (mutation status or treatment failure) only for exposed patients (on prophylaxis or with mutations) were not included because these studies would have biased the analyses by not providing information on unexposed populations for comparison. When more than one article reported on the same study population, only the more comprehensive article was included. From every eligible report, one author extracted information on publication year, study location(s), study start and end dates for calculating data collection calendar midpoint, study size, proportion of HIV-positive patients, number of isolates per patient, timing of prophylaxis in relation to PCP, treatment outcome definition, number and type of DHPS mutations in patients receiving or not receiving sulfa prophylaxis, and sulfa treatment outcome among patients with and without DHPS mutations. Multiple isolates from the same patient were included as independent counts of PCP.

Statistical Analysis

STATA Version 8.2 (Stata Corp., College Station, TX) was used to analyze estimates of the effect of prophylaxis on mutation occurrence and estimates of the effect of mutation on treatment outcome. Both analyses used the risk difference (RD) as the effect measure. Qualitatively similar results were obtained by using the risk ratio and incidence odds ratio (15). The number of patients needed to treat (NNT) to increase or decrease the number of outcomes by one may be computed as $NNT = RD^{-1}$ (16). The 95% confidence limit difference (CLD), computed as the difference between the upper and lower limits of the 95% confidence interval (CI), was used to gauge the precision of the study-specific RD estimates, with smaller values denoting more precise estimates (17). We obtained p values for overall association from the meta-analysis of RD estimates by means of the Mantel-Haenszel test statistic. The potential for publication bias was assessed by visually examining funnel plots of RD estimates and by using standard tests of funnel plot asymmetry (18,19). Homogeneity test statistics and their associated p values were computed to assess the consistency of estimated RDs across studies. Random-effects meta-regression and stratified analyses were used to estimate associations between RD estimates and characteristics of studies and patients. The precision-weighted meta-regression models incorporated random effects by using a restricted maximum likelihood method to estimate the among-study variance (20).

Results

Thirteen eligible studies were identified for the analysis of the effect of prophylaxis on mutation (21–33) and five for the analysis of mutation effect on treatment outcome (Table 1) (25–27,34,35). Three studies were included in both analyses (25–27).

Prophylaxis Effect on Mutation

In this analysis, the estimated RD from each study is the risk of developing a DHPS mutation among PCP patients exposed to sulfa prophylaxis minus the risk among PCP patients not exposed to sulfa prophylaxis. The RD meta-analysis produced strong evidence of a positive association ($p < 0.001$). Twelve of the 13 studies reported results suggesting that prophylaxis increases the risk for DHPS mutations, and 95% CI of 10 of the 13 excluded the null value (Table 1, Figure). The 12 positive RD estimates ranged from a 10% increase in risk (28) to a 69% increase (23). The least precise estimate came from a study with only 20 isolates (26), and the most precise estimate from a study with 236 (29). Visual inspection of the funnel plot, Begg and Mazumdar's test ($p = 0.5$), and the test of Egger et al. ($p = 0.1$) all gave no appreciable evidence of asymmetry. The study-specific results were highly heterogeneous ($p < 0.001$), however. As shown in the Figure, the 95% CI for three estimates (27,29,30) did not overlap the CI for five other estimates (22,23,25,32,33). No single summary estimate can adequately describe results as disparate as these (36).

Of the examined characteristics, data collection calendar midpoint and multiple isolates both had strong associations with the study results (Table 2). Higher estimated RDs were produced by studies in which at least half of the data was collected before 1996 (21–23,25,26,31,32) and from studies including multiple isolates per patients (22,23,26,29,33). Three studies had a data collection calendar midpoint before 1996 and used multiple isolates per patient (22,23,26). The magnitude of the combined influence of these two characteristics on the estimate (difference of RD = 0.10, 95% CLD 0.22) was less than either of the individual characteristics examined singly. Only two studies with a midpoint of 1996 or later included multiple isolates from the same patient (27,28).

The four studies that detailed prophylactic drug use for each specific mutation had a high homogeneity p value (p

Table 1. Study characteristics and effect estimates

Study	Location/data collection calendar midpoint[a]	N	Multiple isolates per patient	Defined prophylaxis timing[b]	Defined treatment outcome[c,d]	Proportion HIV+	RD (95% CI) (95% CLD)[d]
Prophylaxis effect on mutation							
Kazanjian (1998) (21)	USA/1994	27	No	Yes	NA	0.74	0.61 (0.25, 0.97) (0.72)
Helweg-Larsen (22)	Denmark/1994	152	Yes	Yes	NA	1.00	0.51 (0.33, 0.70) (0.37)
Ma (1999) (23)	USA/1992	37	Yes	Yes	NA	0.70	0.69 (0.43, 0.94) (0.51)
Huang (24)	USA/1998	111	No	Yes	NA	1.00	0.33 (0.15, 0.51) (0.36)
Kazanjian (2000) (25)	USA/1995	97	No	Yes	NA	1.00	0.52 (0.35, 0.70) (0.35)
Visconti (26)	Italy/1995	20	Yes	No	NA	1.00	0.60 (0.20, 1.00) (0.80)
Ma (2002) (27)	Italy/1998	107	No	Yes	NA	1.00	0.15 (0.01, 0.30) (0.29)
Costa (28)	Portugal/1998	89	No	Yes	NA	0.93	0.10 (–0.15, 0.35) (0.50)
Crothers (29)	USA/2000	236	Yes	Yes	NA	1.00	0.16 (0.06. 0.25) (0.19)
Latouche (30)	France/2000	92	No	Yes	NA	0.90	–0.03 (–0.22, 0.16) (0.38)
Miller (31)	England/1993	25	No	Yes	NA	1.00	0.31 (–0.08, 0.69) (0.77)
Nahimana (32)	France/1995	158	No	Yes	NA	0.76	0.50 (0.31, 0.69) (0.38)
Zingale (33)	Italy/1999	64	Yes	Yes	NA	1.00	0.61 (0.42, 0.80) (0.38)
Mutation effect on treatment outcome							
Kazanjian (2000) (25)	USA/1995	97	No	NA	Yes	1.00	0.22 (0.01, 0.43) (0.42)
Takahashi (34)	Japan/1997	24	No	NA	Yes	0.67	0.89 (0.59, 1.19) (0.60)
Ma (2002) (27)	Italy/1998	107	No	NA	NA	1.00	–0.01 (–0.22, 0.20) (0.42)
Navin (35)	USA/1997	136	No	NA	Yes	1.00	–0.21 (0.39, –0.03) (0.36)
Visconti (26)	Italy/1995	20	Yes	NA	No	1.00	–0.21 (–0.82, 0.40) (1.22)

[a]Data collection calendar midpoint, the midpoint in calendar time of data collection.
[b]Defined prophylaxis timing, whether the study stated the timing of prophylaxis in relation to the episode of *Pneumocystis jirovecii* pneumonia.
[c]Defined treatment outcome, whether the study stated how it defined treatment outcome.
[d]RD, risk difference; CI, confidence interval; CLD, confidence limit difference; NA, not applicable.

= 0.6) and a higher estimated RD (Table 2) (22,26,31,33). One study did not provide information on the timing of sulfa prophylaxis in relation to the PCP episode (26). With this study removed so its influence on the meta-analysis could be evaluated, the homogeneity p value remained low for the other 12 studies ($p < 0.001$). The remaining characteristics were weakly associated with study results.

Mutation Effect on Treatment Outcome

In this analysis, the estimated RD from each study is the risk of failing sulfa treatment for PCP among patients with DHPS mutations minus the risk among patients without DHPS mutations. Five studies provided such a result. One of these studies had a mixed HIV-positive and HIV-negative patient population (34), and another did not describe the criteria for determining treatment outcome (26). Three of the studies included in the analysis of prophylaxis effect on mutation (22,30,32) mentioned examining treatment outcome but did not provide usable treatment outcome data for the full study population.

Assessing publication bias was impractical with only five published studies. Two of the five suggested that patients infected with mutant *P. jirovecii* were unexpectedly more likely to be responsive to treatment for PCP (Table 1) (26,35). One study showed that mutations had virtually no effect (27). The remaining two studies were on the opposite side of the null hypothesis (Table 1) (25,34). The pronounced evidence of heterogeneity ($p < 0.001$) was easily discerned by examining CI nonoverlap, since the 95% CI for the study with the highest estimate for increased risk(34) did not overlap any of the other four CIs.

Discussion

PCP patients receiving sulfa prophylaxis are at increased risk for DHPS mutations compared with PCP patients not receiving sulfa. The strength of the association

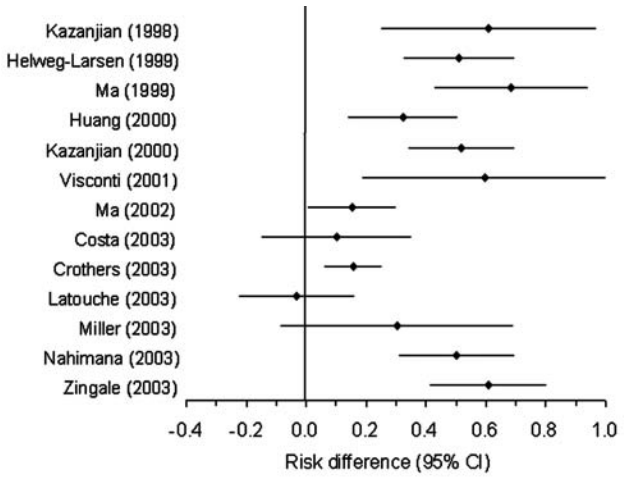

Figure. Forest plot, prophylaxis effect on mutation. CI, confidence intervals.

varies greatly across studies.

Not all studies adhered to a uniform definition of substantive sulfa exposure. Only some defined a minimum duration of prophylaxis use, often in conjunction with the timing of the PCP episode, for a patient to be counted as receiving prophylaxis. Some studies were more comprehensive in documenting prophylactic drug use by pulling pharmacy records to verify that prophylactic medications were dispensed or patient questionnaires to confirm that the drug was taken. Moreover, the association between prophylactic drug use and mutation was stronger for the studies that included multiple isolates than for those that did not. This difference suggests the possibility that exposure to multiple courses of sulfa prophylaxis increases the chance of developing DHPS mutations. The weakened association evident since 1996 may reflect a higher overall prevalence of mutation with a higher prevalence among those unexposed to prophylaxis, or it may reflect that fewer HIV-infected patients take prophylactic drugs because of HAART. Each of these factors may bear on the strength of the association between sulfa prophylaxis and DHPS mutations. Variations in unreported aspects of study design or patient characteristics may account for the remaining inconsistency in estimated effect size.

One of the 13 studies reported an inverse association between prophylaxis and mutations (30). Unlike the other studies, this study categorized prophylaxis use as regular, irregular, none, and unknown. We categorized sulfa exposure as regular or irregular use. Had we counted only regular prophylactic drug use as sulfa exposure, the association in this study would have been positive, albeit very imprecise (RD = 0.17, 95% CLD = 1.32). Additionally, the isolates in this study were collected more recently than in other studies, with all specimens collected after 1998.

This systematic review was unable to resolve the conflicting results regarding the magnitude of the effect of DHPS mutations on treatment outcome. Only five studies were eligible for inclusion in this analysis. Although the small number of studies precluded a statistical investigation of possible explanations for the inconsistent findings, variation in definitions of treatment outcome may be partially responsible. The two studies with positive associations used clinical improvement after therapy to determine

Table 2. Stratified and random-effects meta-regression analysis of study characteristics

Study characteristic[a]	Characteristic level	No. of studies	RD (95% CLD)[b]	Homogeneity test p value	Difference of RDs (95% CLD)[b]
Prophylaxis effect on mutation					
Data collection calendar midpoint	1996 or later	6	0.22 (0.31)	< 0.001	–0.32 (0.39)
	Before 1996	7	0.53 (0.18)	0.8	0
	4-y change	13	NA	NA	–0.23 (0.30)
Prophylaxis use by Specific mutations	Yes	4	0.54 (0.24)	0.6	0.20 (0.55)
	No	9	0.32 (0.29)	< 0.001	0
Multiple isolates per patient	Yes	5	0.50 (0.50)	< 0.001	0.19 (0.52)
	No	8	0.30 (0.32)	< 0.001	0
Location	USA	5	0.44 (0.43)	< 0.001	0.10 (0.55)
	Outside USA	8	0.34 (0.36)	< 0.001	0
Defined treatment outcome	Yes	5	0.32 (0.44)	< 0.001	–0.08 (0.54)
	No	8	0.41 (0.35)	< 0.001	0
Multicenter	Yes	5	0.41 (0.31)	0.0	0.05 (0.55)
	No	8	0.36 (0.36)	< 0.001	0
Proportion HIV+	1.00	8	0.38 (0.30)	< 0.001	0.03 (0.56)
	< 1.00	5	0.37 (0.58)	< 0.001	0

[a]Data collection calendar midpoint, the midpoint in calendar time of data collection; defined treatment outcome, whether the study stated how it defined treatment outcome.
[b]RD, risk difference; CLD, confidence limit difference; NA, not applicable.

treatment outcome (25,34), whereas the two studies with negative associations defined treatment outcome as survival after the episode (26,35). The treatment outcome definition for the study showing minimal effect used both survival and clinical recovery without relapse (27). HIV status may also have swayed the results. The sole study to include HIV-uninfected patients noted the strongest association between mutation and treatment outcome (34).

This systematic review has both strengths and limitations. It included information on all relevant studies for which results have been reported, examined how different study characteristics influenced the magnitude of effect estimates, and provided information that may be useful when designing future studies of a similar nature. Its principal weakness was that the small number of available studies, especially for treatment outcome, made the results from stratified analysis and meta-regression less precise than would be desirable.

We conclude that exposure to sulfa prophylaxis for PCP increases the risk for DHPS mutations. This finding is evident even with the heterogeneity of the individual study results. Although whether these mutations are clinically relevant is unclear, they are likely to develop in patients who have received sulfa prophylaxis for PCP for extended periods. This review did not clarify the effect of these mutations on treatment outcome. Further studies are needed to examine the association between DHPS mutations and treatment outcome in patients with PCP. Until these studies are performed, the optimal treatment for patients with PCP, who have had substantive exposure to sulfa prophylaxis and who are therefore likely to have DHPS mutations, remains speculative.

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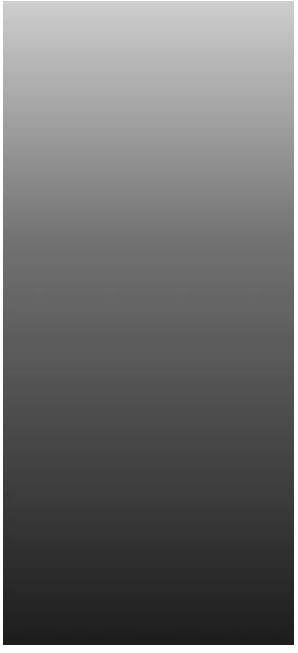

Molecular Evidence of Interhuman Transmission of *Pneumocystis* Pneumonia among Renal Transplant Recipients Hospitalized with HIV-Infected Patients

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Ten *Pneumocystis jirovecii* pneumonia (PCP) cases were diagnosed in renal transplant recipients (RTRs) during a 3-year period. Nosocomial transmission from HIV-positive patients with PCP was suspected because these patients shared the same hospital building, were not isolated, and were receiving suboptimal or no anti-PCP prophylaxis. *P. jirovecii* organisms were typed with the multitarget polymerase chain reaction–single-strand conformation polymorphism method. Among the 45 patients with PCP hospitalized during the 3-year period, 8 RTRs and 6 HIV-infected patients may have encountered at least 1 patient with active PCP within the 3 months before the diagnosis of their own PCP episode. In six instances (five RTRs, one HIV-infected patient), the patients harbored the same *P. jirovecii* molecular type as that found in the encountered PCP patients. The data suggest that part of the PCP cases observed in this building, particularly those observed in RTRs, were related to nosocomial interhuman transmission.

Pneumocystis jirovecii pneumonia (PCP) is a severe opportunistic infection in immunocompromised patients (1,2). It remains a major problem in some HIV-infected persons who are not receiving or not responding to highly active antiretroviral triple therapy and among those who are unaware of their HIV status. PCP is also of clinical importance in immunosuppressed patients, e.g., transplant recipients and those receiving chemotherapy for malignant diseases, who are not infected with HIV. Host specificity suggests that the reservoir of *P. jirovecii* is limited to humans. Primary infection in infants (3,4), as well as asymptomatic carriage by immunosuppressed persons (5–8), may serve as infectious reservoirs or sources in the community. Reactivation of a past infection was a postulate mechanism of infection in immunosuppressed patients, but de novo infection in recurrent episodes of the disease (9) has suggested that infection or reinfection from exogenous sources may occur. Horizontal airborne transmission has been demonstrated in several animal models (10–12).

Transmission of *P. jirovecii* from patients with active PCP to susceptible persons has been suspected in numerous descriptions of nosocomial clusters of PCP cases (13–17). Although a common environmental source of the infection was difficult to exclude, many patients in the clusters had contact with each other, which suggests that they may have transmitted *P. jirovecii* to one another. The early reports of PCP epidemics among malnourished children in orphanages and hospitals in the 1950s were also compatible with interhuman transmission of *P. jirovecii* (18). The strongest suspicion of transmission was provided by a case-control study performed for a cluster of five PCP cases in transplant recipients (13). This analysis

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showed that the patients had more encounters than matched controls at the outpatient facility with HIV patients who had or subsequently developed PCP. However, in these studies, transmission of *P. jirovecii* could not be assessed at the molecular level because no molecular typing method for *P. jirovecii* existed. Such methods were developed in the 1990s, and new clusters were analyzed. However, the few published anecdotal analyses often reported different genotypes within the clusters (19–21). Thus, interhuman transmission of *P. jirovecii* from PCP cases is still an open issue.

The latest guidelines developed by the U.S. Public Health Service and the Infectious Diseases Society of America for preventing opportunistic infections in persons infected with HIV state that although some authorities recommend that persons who are at risk for *P. jirovecii* pneumonia not share a hospital room with a patient who has PCP, data are insufficient to support this recommendation as standard practice (22,23).

In our molecular epidemiologic study, we investigated the possibility of *P. jirovecii* transmission between persons during a 3-year period in a hospital building that simultaneously hosted HIV patients (with and without PCP) and renal transplant recipients (RTR) (often during rejection episodes), and in which a cluster of PCP was observed.

Material and Methods

Hospital Setting

Edouard-Herriot Hospital is a 1,200-bed healthcare facility in Lyon, France, and is made up of several buildings. One of these buildings (building A, 80 beds) accommodates one hospital ward, an intensive care unit, an outpatient clinic, and a radiodiagnostic facility, which are mostly devoted to renal transplant medicine and clinical immunology, including HIV medicine. Another building (building B) hosts only patients with hematologic malignancies and is located 100 m away from building A.

Data Collection

Our investigation included the 39 patients with PCP who were hospitalized in building A and whose bronchoalveolar lavage (BAL) specimen was available for molecular typing. These patients were chosen because interhuman transmission of *P. jirovecii* was suspected in this building. The database of the Department of Medical Information of the University Hospitals of Lyon was used to identify the demographic and clinical characteristics of the patients. Relevant data (prophylaxis regimen, hospitalization periods, dates of outpatient visits, immunosuppressive regimen) were also extracted from the medical charts of patients by using a questionnaire and log books of the outpatient clinic and radiodiagnostic facility.

Laboratory Diagnosis and Storage of Specimens

PCP was diagnosed by using methenamine-silver nitrate (24) and Giemsa stains on BAL specimens in the parasitology laboratory of Claude-Bernard University, which has processed all specimens using the same techniques for many years. The number of BAL specimens submitted for patients seen at Edouard-Herriot Hospital has been stable over the years (1992–1998: 235, 241, 277, 254, 290, 215, 215, respectively). BAL specimens of patients with proven PCP were stored at –20°C.

Molecular Typing

BAL specimens were typed as described previously (25–27) with the polymerase chain reaction (PCR)–single-strand conformation polymorphism (SSCP) method for typing *P. carinii*, now named *P. jirovecii* (28), in humans. The method consists of amplifying four variable regions of the *P. jirovecii* genome, followed by the detecting the polymorphisms with SSCP. The variable regions analyzed are the internal transcribed spacer 1 of the nuclear rDNA operon, the intron of the nuclear 26S rRNA gene, the variable region of the mitochondrial 26S rRNA gene, and the region surrounding the intron 6 of the β-tubulin gene. The different SSCP patterns observed are caused by one to four base-pair polymorphisms (26). A *P. jirovecii* type is defined by a combination of four alleles, which corresponds to the four genomic regions. If a specimen harbors two alleles of one or more of the four genomic regions, the patient was considered coinfected with two or more *P. jirovecii* types (25). For a given patient, each type is defined as an "isolate." Molecular typing was performed on specimens from patients in building A from 1994 to 1996, as well as on representative specimens collected during the same period in building B of the Edouard-Herriot hospital and in other university hospitals of Lyon. In addition, the dihydropteroate synthase (DHPS) genotype was determined by using PCR-SSCP as described (29). Four DHPS alleles have been described in *P. jirovecii* (30). The mutated alleles result in an amino acid change in the active site of the enzyme at position 55 (allele M1) or 57 (M2), or both polymorphisms (M3).

Definitions

In the absence of knowledge of many biologic and epidemiologic characteristics of *P. jirovecii* infection, the incubation period of the not yet symptomatic patients and the period of infectivity of patients with PCP were postulated on the basis of available human and experimental data. Described clusters of PCP (13,14) suggest that the incubation period of de novo infection is 3–12 weeks. Accordingly, we assumed that a new infection with *P. jirovecii* (as opposed to reactivation) would occur 3–12 weeks before laboratory diagnosis of PCP. This finding is

also in accordance with experiments in animals (31–33). Similarly, we considered that the risk of transmission from a *P. jirovecii*-infected patient to a susceptible one was likely to be highest from early symptoms to the middle of treatment. We assumed that an infected patient could transmit *P. jirovecii* from 3 weeks before to 2 weeks after the PCP diagnosis. We hypothesized that transmission was airborne and defined that a potentially infectious encounter occurred if a patient within his or her susceptible period and another patient within his or her infectious period visited the same location in building A on the same day. Transmission was considered possible if the patients encountered at least once and shared a common *P. jirovecii* type.

PCP Prophylaxis, Isolation, and Immunosuppression

Four HIV-infected patients and four transplant recipients at risk for PCP were receiving sulfadoxine-pyrimethamine, but at a dosage lower than recommended for anti-*P. jirovecii* prophylaxis (25 mg pyrimethamine plus 500 mg sulfadoxine in one tablet taken once a week or every 2 weeks versus two tablets per week [34]). In addition, four HIV-infected patients were receiving aerosolized pentamidine (300 mg every 2 weeks). The other 27 patients did not receive any anti-*Pneumocystis* prophylaxis. A policy for isolating patients according to their underlying disease or to the occurrence of a PCP episode did not exist. Patients were allowed to move freely in the units and shared a TV room when permitted by their general condition. All RTRs, including those who experienced PCP, received usual inductive and maintenance treatments with prednisone, azathioprine, and cyclosporine. Treating rejection included high-dose corticosteroids and, if necessary, monoclonal antibodies.

Case-Case Comparison

Case-case comparison based on molecular typing of the pathogen (35) was performed. Groups of cases infected with different *P. jirovecii* molecular types were compared to investigate differences in exposure histories.

Results

From 1994 to 1996, a total of 45 patients with 46 episodes of PCP were hospitalized in building A of the Edouard-Herriot Hospital. Their age ranged from 23 to 56 years (median 41), and most of them were male (82%). Thirty-six episodes were observed in 35 HIV-infected patients and 10 episodes in 10 RTRs. Thirty-one HIV-infected patients were admitted because of PCP, and PCP developed in all 10 RTRs and 4 HIV-infected patients during or shortly after hospitalization. These numbers represented a substantial increase compared to previous years, particularly in RTRs in whom only one case had been diagnosed during the 7 preceding years (Figure 1; the likelihood ratio for equality of two Poisson processes yields p = 0.00002; 1 case in 7 years versus 10 in 3 years). During the period from 1993 to 1996, the number of hospital patient-days for RTRs decreased by 37%, whereas those of the populations of HIV-infected patients and of patients with PCP increased, respectively, by 36% and 63% (Figure 1). The number of admissions of HIV-infected patients in building A was 339 in 1993, 364 in 1994, 401 in 1995, and 445 in 1996. The number of admissions of RTRs was 469 in 1993, 311 in 1994, 319 in 1995, and 297 in 1996. Precise admission figures before 1993 are not available, but the number of renal transplants performed at Edouard-Herriot Hospital has been decreasing from 128 in 1987 to 74 in 1996 and has been stable since then (Figure 1). The immunosuppressive regimen has not changed for RTRs from 1990 to 1997 in Edouard-Herriot Hospital.

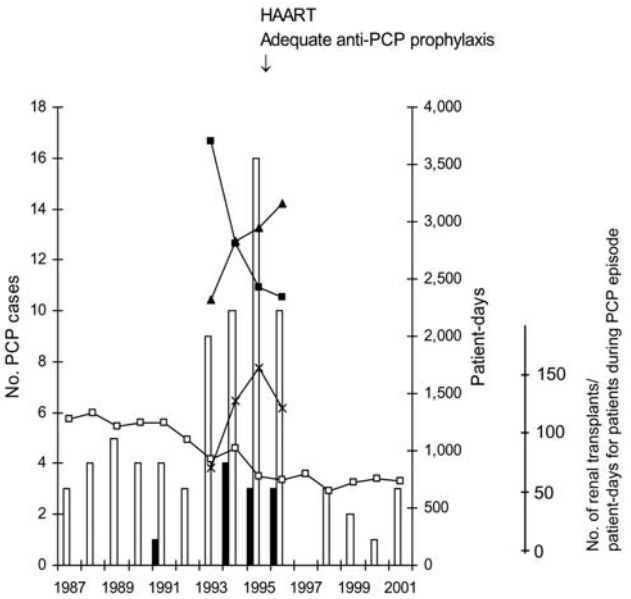

Figure 1. *Pneumocystis jirovecii* pneumonia (PCP) cases in HIV-infected patients (white bars) and in transplant recipients (black bars) at building A of Edouard-Herriot Hospital. Solid lines show the number of hospital patient-days for transplant recipients (filled squares), for HIV-infected patients (filled triangles), and for the patients during their PCP episode (crosses), as well as the number of renal transplantations performed (white squares). HAART, highly active antiretroviral therapy.

Molecular Typing *P. jirovecii*

Thirty-nine of the 46 BAL specimens collected from 1994 to 1996 were available for typing (30 in HIV-infected patients and 9 in transplant recipients). Nineteen (49%) specimens corresponded to an infection with a single *P. jirovecii* type, 15 (38%) with two types, and 5 (13%) with more than two types. A total of 19 different *P. jirovecii* types were observed. In building A, the frequency of each

type was 2%–12% of the *P. jirovecii* isolates, except for type 1 which represented 39% of the isolates and was isolated in 19 patients. Type 1, the most prevalent, represented 10%–20% of the isolates in Switzerland and other European cities (27), as well as in building B and other hospital facilities of Lyon (Figure 2). In particular, the frequency of type 1 was significantly higher in building A than in the other hospital facilities of Lyon (19 of 45 versus 28 of 145, Fisher exact test p = 0.003). Moreover, the frequency distribution of type 1 in the different categories of PCP patients hosted in building A was significantly different: it represented 31% (12 of 39) of the isolates from the HIV-infected patients, but 70% (7 of 10) of those from the transplant recipients (Figure 3, Fisher exact test p = 0.033). Seven of the 12 HIV-infected patients infected with *P. jirovecii* type 1 also harbored another type (coinfection), whereas one of the seven transplant recipients had a coinfection.

Encounters and Exposures between Patients with *P. jirovecii*

From 1994 to 1996, 14 of 39 patients with PCP and available BAL specimens had prior encounters with patients with PCP (6 of the 30 HIV-infected patients and 8 of the 9 transplant recipients). A total of 118 potential encounters between patients with active PCP and patients who developed PCP 3–12 weeks after the encounter could be retrieved (Table). These 118 encounters corresponded to one or several encounters for the 14 patients. Among these 14 patients, PCP developed in 6 due to the same *P. jirovecii* type as 1 or 2 encountered PCP source patients, and *P. jirovecii* type 1 was involved in all 6 patients (5 transplant recipients, 1 HIV-infected patient). Of the 80 exposures involving *P. jirovecii* type 1, six PCP episodes were observed compared to no episodes of 38 exposures not involving *P. jirovecii* type 1.

Figure 4 shows the characteristics and chronologic events of the six putative nosocomial cases and their presumed source patients. It also shows the DHPS genotype. Five of the six nosocomial PCP patients harbored the M2 mutation and one the M3 mutation. Three nosocomial PCP patients harboring M2 mutation were receiving suboptimal anti-PCP prophylaxis. In all nosocomial cases, the presumed source patients had the same DHPS genotype. In some cases, an additional *P. jirovecii* genotype was recovered from the source patient, a finding compatible with coinfection with two types, one of which was not transmitted or present in a proportion sufficient to be detected in the nosocomial case. All the nosocomial PCP episodes in RTRs followed encounters which occurred when they were strongly immunosuppressed because of the treatment of a rejection episode (Figure 4).

Case-case comparison was used to compare exposure histories of two groups of patients, those harboring type 1 and those who did not. The proportion of patients who had at least one encounter during their susceptible period with a patient with active PCP harboring type 1 was higher in the first group (6/19 vs. 2/20), although not significant (p = 0.13, Fisher exact test). The frequency of the M2 mutation in patients not receiving any sulfa prophylaxis

Table. Cases with *Pneumocystis jirovecii* pneumonia (PCP) and potential encounters 3 weeks to 3 months before their PCP episode with other patients with active PCP

Date of PCP	Underlying disease[a]	*P. jirovecii* PCR-SSCP type	CD4 counts/mm^3	No. of encounters with patients with active PCP	No. patients with active PCP encountered		Presumptive nosocomial PCP[b]
					Total	With same *P. jirovecii* type	
1/10/94	RTR	1 and 6	–[c]	10	1	0	No
1/11/94	RTR	23	–	20	1	0	No
11/5/94	RTR	Undetermined	–	5	1	0	No
12/6/94	HIV	6	0	2	2	0	No
12/6/94	RTR	1	–	5	3	1	Yes
12/13/94	HIV	6 and 7	0	10	1	0	No
1/31/95	HIV	13 and 26	67	1	1	0	No
9/20/95	HIV	1	4	16	2	0	No
10/20/95	HIV	1	18	1	1	0	No
12/28/95	RTR	1	–	9	1	1	Yes
2/22/96	RTR	1	–	14	3	2	Yes
2/28/96	HIV	1	0	14	2	1	Yes
5/22/96	RTR	1	–	8	7	1	Yes
5/23/96	RTR	1	–	3	3	1	Yes
Total	8 RTRs, 6 HIV			118			5 RTR, 1 HIV

[a]PCR, polymerase chain reaction; RTR, renal transplant recipient; SSCP, single-strand conformation polymorphism.
[b]Nosocomial PCP was defined as PCP episode with exposure to one or several source patients infected with the same *P. jirovecii* type 3–12 weeks before the diagnosis.
[c]Data not available.

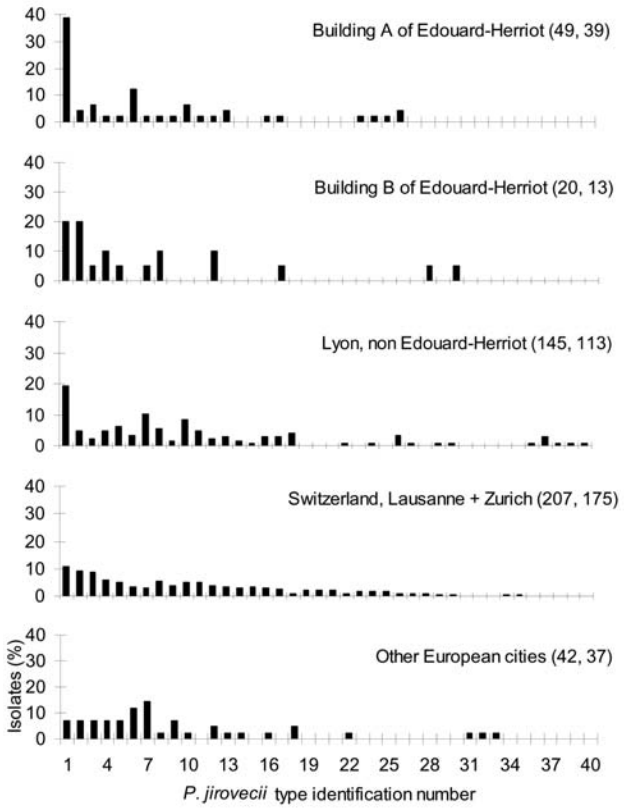

Figure 2. Frequency distribution of *Pneumocystis jirovecii* types observed in different cities and hospitals. Each type was considered as one isolate. The number of isolates followed by the number of specimens analyzed are indicated in the parenthesis for each geographic location. Data from Switzerland and other European cities are reproduced with permission from Hauser et al. 2001, AIDS 15(4):461–6 (27).

was significantly higher in building A than in other hospital facilities of Lyon (10/27 vs. 6/85, χ^2 test p = 0.0004).

Intervention

By mid-1996, all susceptible patients were placed on appropriate prophylaxis with co-trimoxazole (sulfamethoxazole plus trimethoprim), and HIV-infected patients had been started on highly active antiretroviral therapy. No PCP case was observed in 1997 (Figure 1), and no PCP cases were observed in transplant recipients as of December 2003.

Discussion

During a 3-year period, 10 cases of PCP in transplant recipients occurred in a building of the Edouard-Herriot Hospital in Lyon, whereas only one case was observed in the preceding 7 years. These cases could not be attributed to improved diagnosis, change of immunosuppression regimen, an increase (a decrease actually occurred) of transplant recipients hospitalized in the facility. However, the outbreak occurred concomitantly with a progressive increase in the number of HIV-infected patients with and without PCP hospitalized in the same facility. Thorough molecular and epidemiologic analyses of the PCP cases showed the following facts: 1) transplant recipients, often in a stage of severe immunosuppression, shared the facility with HIV-infected patients with and without active PCP; 2) both transplant recipients and HIV-infected patients were receiving no or suboptimal anti-PCP prophylaxis; 3) *P. jirovecii* type 1 represented 70% of the isolates from the transplant PCP cases, but it represented ≤31% of the isolates in the HIV-infected patients with PCP in Lyon and elsewhere; and 4) all the transplant recipients (and some HIV patients) in whom PCP developed had been hospitalized in the facility at some point during the 3 months preceding their PCP episodes. Moreover, the proportion of patients exposed to *P. jirovecii* type 1 during their susceptible period was higher among cases infected with type 1 than among those not harboring type 1, although it did not reach statistical significance (p = 0.05). Finally, review of the medical charts indicated potential encounters between PCP cases during their susceptible period and other patients with active PCP (Table). Encounters with patients with a PCP episode involving the same *P. jirovecii* type, which may have led to transmission, were possible in 5 of the 10 transplant recipients and 1 of the 30 HIV patients. Taken together, these facts suggest that at least half of the PCP cases in transplant recipients (and possibly some in HIV patients) may be the result of a nosocomial acquisition of *P. jirovecii*.

However, alternative explanations exist that cannot be excluded. First, the transient presence of *P. jirovecii* in the

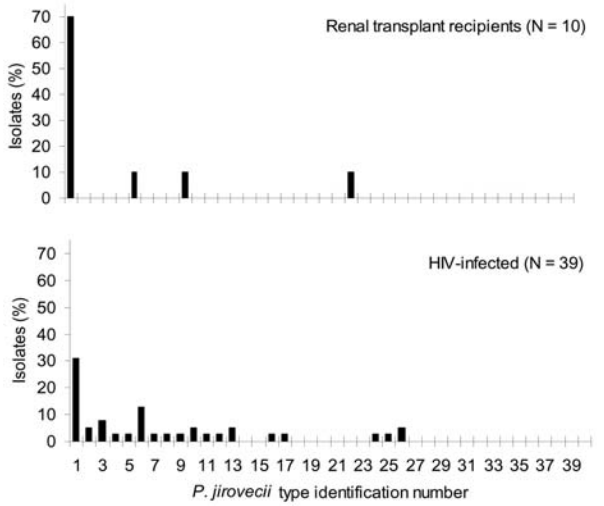

Figure 3. Frequency distribution of *Pneumocystis jirovecii* types observed in 30 HIV-infected patients and nine renal transplant recipients from 1994 through 1996 at building A of the Edouard-Herriot Hospital.

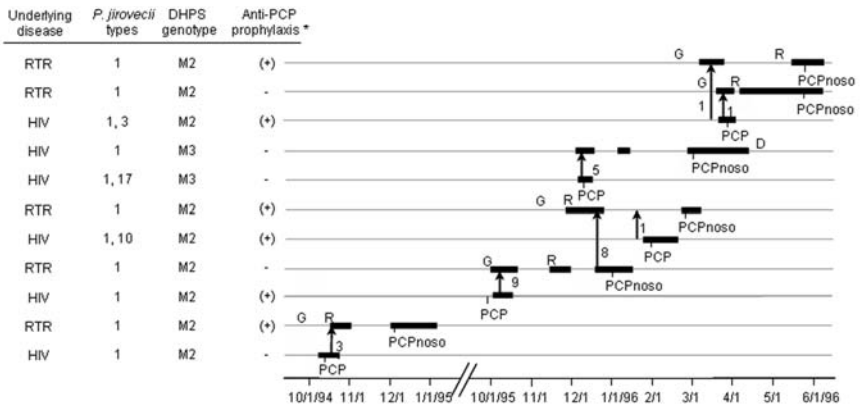

Figure 4. Potential encounters compatible with nosocomial interhuman transmission of *Pneumocystis jirovecii* at building A of the Edouard-Herriot Hospital (see Methods). Thicker parts of solid lines represent periods of hospitalization. Each encounter or consecutive encounters are figured by an arrow with the head indicating the direction of the presumed transmission, the number of encounters being indicated close to each arrow. *Anti-PCP prophylaxis was suboptimal. D, death. G, graft. R, rejection episode. RTR, renal transplant recipient. PCPnoso, nosocomial case.

air of hospital corridors has been described (36), which raises the possibility of an environmental source of *P. jirovecii* type 1 in building A. The following facts argue against this possibility: 1) the existence of a long-lasting environmental source of *P. jirovecii* has never been established, and 2) a high prevalence of type 1 was not observed in building B, which is located 100 m from building A and hosts patients with hematologic malignancies. Second, our study provides epidemiologic and molecular evidence that nosocomial transmission of *P. jirovecii* can occur, but whether this transmission would have occurred directly or indirectly through carriers is unclear. Indeed, carriage of *P. jirovecii* DNA has been described in the lungs of asymptomatic, immunosuppressed persons (5–8), as well as in the nose of immunocompetent relatives and healthcare workers in close contact with a PCP patient (37). Moreover, transmission by immunocompetent carriers to susceptible hosts has been demonstrated in the mouse model (38). Thus, indirect transmission through healthcare workers, physicians, or asymptomatic immunosuppressed patients cannot be ruled out.

For 14 of the 39 patients with PCP observed during the 3-year period, potential encounters with patients with active PCP during the 3 months preceding their episode had been documented (Table). However, only *P. jirovecii* type 1 was involved in encounters that apparently resulted in secondary cases. Part of this observation may be related to the higher prevalence of type 1 in Lyon (≈20%), although this could not explain the 40% rate of type 1 in building A. Another possibility is that type 1 might be more transmissible or virulent. This finding would be consistent with the fact that this type was one of the most prevalent types also in other geographic areas (Figure 2) (27). Moreover, specific *P. jirovecii* genotypes have been associated with more severe clinical symptoms (39) or with resistance to certain drugs (40). In our study, a mutation in the active site of DHPS was present in all six presumptive nosocomial PCP cases. The mutation may have favored acquisition of type 1 rather than another type by the three patients who were receiving suboptimal prophylaxis with Fansidar (Roche, Nutley, NJ). The presence of this mutation in the nosocomial PCP cases of our study suggests that *P. jirovecii* was acquired shortly before the episode because the frequency of DHPS mutations greatly increased only in the 1990s (41). Moreover, in patients not receiving any sulfa prophylaxis, the frequency of M2 mutation was significantly higher in building A than in other hospital facilities of Lyon ($p = 0.0004$), a fact that suggests nosocomial interhuman transmission of *P. jirovecii*.

Our study provides insight into the relative importance of nosocomial acquisition of *P. jirovecii* if infectious and susceptible patients are in close contact. Even though infected and susceptible patients were kept in unusually close proximity in this hospital, relatively few cases compatible with nosocomial interhuman transmission seem to have occurred. This finding suggests that transmission from patients with active PCP is limited, which is consistent with studies that we performed in HIV outpatient clinics that suggested infrequent cross-infections (27,42), as well as with a study comparing contact histories of patients with or without PCP (43). The source remains undetermined for the infection in the five transplant recipients for whom no potentially infectious encounters were found. One possibility is that carriers of *P. jirovecii* in the hospital have played a role.

The available data and the arbitrary definitions we had to use, in light of the absence of precise scientific data on *P. jirovecii* infection, are limitations of our study. We could not demonstrate that the presumed encounters actually occurred or define the precise nature of the encounter. Also, we could not firmly exclude other potential sources of *P. jirovecii*, such as the environment or asymptomatic carriers. Nevertheless, to our knowledge, this study is the first to suggest that *P. jirovecii* may be nosocomially transmitted and acquired by severely immunosuppressed patients. Given the increased number of reports relating resistance to anti-*Pneumocystis* drugs, prophylaxis of

patients at risk might not be sufficient to achieve prevention. Moreover, prophylaxis is often not satisfactory because of secondary effects. Consequently, avoiding contact between persons at risk for PCP and patients with active PCP may be warranted and should be added to prevention guidelines.

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Laboratory Diagnosis of Four Recent Sporadic Cases of Community-acquired SARS, Guangdong Province, China

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Four cases of severe acute respiratory syndrome (SARS) that occurred from December 16, 2003, to January 8, 2004, in the city of Guangzhou, Guangdong Province, China, were investigated. Clinical specimens collected from these patients were tested by provincial and national laboratories in China as well as members of the World Health Organization SARS Reference and Verification Laboratory Network in a collaborative effort to identify and confirm SARS-associated coronavirus (SARS-CoV) infection. Although SARS-CoV was not isolated from any patient, specimens from three patients were positive for viral RNA by reverse transcription–polymerase chain reaction assay and all patients had detectable rises in SARS-CoV–specific antibodies. This study shows the effectiveness of a collaborative, multilaboratory response to diagnose SARS.

In November 2002, cases of a highly contagious and severe atypical pneumonia were identified in Guangdong Province in southern China (1). By March 2003, the infection had spread to Hong Kong, Singapore, Vietnam, Taiwan, Canada, and the United States. Designated severe acute respiratory syndrome (SARS), the outbreak was subsequently linked to infection with a previously unrecognized coronavirus (SARS-CoV) (2–4). The outbreak ended with the World Health Organization (WHO) announcement on July 5, 2003, that the last patient had recovered and the human chain of SARS transmission was broken (5). In all, 29 countries reported to WHO >8,000 SARS cases with 774 deaths (6).

Since WHO's declaration of the end of the SARS epidemic, 17 cases of laboratory-confirmed SARS-CoV infection have been reported. Infections in 13 patients were associated with laboratories: 6 exposed while working in the laboratory and 7 contacts of a patient with a laboratory-acquired case (7–9). The other four cases were acquired in the community. On December 26, the Chinese Ministry of Health informed WHO of the first case of community-acquired SARS in Guangzhou, the capital city

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of Guangdong Province. This case was rapidly followed by reports of three additional cases, all linked to a region in Guangzhou (10). Although none of these cases was fatal or resulted in documented secondary transmission, they demonstrate that community-acquired infection with SARS-CoV, and potential reemergence of SARS leading to epidemic spread, remains a possibility. These cases also highlight some issues associated with diagnosing and confirming the diagnosis of SARS-CoV infection. In this report we describe the laboratory diagnosis and associated confirmation of SARS-CoV infection for these four cases.

Materials and Methods

Patients and Specimens

From December 16, 2003, to January 8, 2004, four SARS cases were identified in Guangzhou, Guangdong Province, China. Patient 1 was a 32-year-old male television producer in whom fever and headache developed on December 16, 2003. Patient 2 was a 20-year-old female waitress who became ill on December 26. Patient 3 was a 35-year-old male businessman in whom fever developed on December 30. Patient 4 was a 40-year-old male hospital director and practicing physician who became ill on January 8, 2004. All patients had temperatures >38°C and x-ray evidence of pneumonia. A confirmed case of SARS is defined as clinically compatible illness with laboratory-confirmed evidence of SARS-CoV infection. Initial tests by the Center for Disease Control and Prevention of Guangdong Province and the Center for Disease Control and Prevention of Guangzhou (laboratory A; laboratories that participated in this study are listed in Table 1) were positive for SARS-CoV, which prompted a systematic collection of clinical specimens, including respiratory secretions, urine, stool, and serum collected at different time points in patients' illnesses. Confirmatory testing was performed by the Institute for Viral Disease Control and Prevention and Institute for Communicable Infection Disease, China Center for Disease Control (laboratory B) and members of the WHO SARS Reference and Verification Laboratory Network (11), including the Government Virus Unit and Department of Microbiology, Queen Mary Hospital, Hong Kong Special Administrative Region (laboratory C), and the U.S. Centers for Disease Control and Prevention (laboratory D).

Serologic Tests

SARS-CoV Microneutralization Assay

Serum specimens were tested by laboratories B, C, and D for neutralizing antibodies to SARS-CoV by microneutralization assay using the procedure of Sui et al. (12) but with different virus strains (laboratory D, Urbani strain; laboratory B, P9 and P11; laboratory C, strain 6109 or HKU-39846). Briefly, serial dilutions were prepared and added in triplicate to 96-well plates (Costar, Corning, NY). Approximately 75 PFU of SARS-CoV was added to the diluted serum samples and incubated at 37°C for 45 min. Vero E6 cells (2×10^5/mL) were added to the wells, and the mixture was incubated at 37°C for 3 to 4 days. Results were visualized by staining the wells with a crystal violet–formaldehyde staining reagent (0.013% crystal violet, 2.5% ethanol, and 10% formaldehyde in 0.01 mol/L phosphate-buffered saline [PBS]) for 1 h at room temperature. The neutralization titer was measured as the reciprocal of the highest serum dilution that completely inhibited Vero E6 cell lysis in at least two of the three triplicate wells.

SARS-CoV Enzyme Immunoassay

Laboratories A, B, and C performed serologic testing for SARS-CoV–specific antibodies by using an indirect enzyme immunoassay (EIA) kit (Beijing BGI-GBI Biotech Co., Beijing, China) in accordance with the manufacturer's instructions. Briefly, diluted serum specimens were added to SARS-CoV lysate–coated wells and incubated at 37°C for 30 min. The wells were rinsed with a wash solution and incubated with a conjugated antibody solution at 37°C for 30 min. After the washing, a substrate solution was added to wells and incubated at 37°C for 10 min in the dark. Termination solution was added and the optical density (OD) measured using 450 nm with a reference wavelength at 630 nm. For every assay, one blank control, one positive control, and two negative control wells were included. The cutoff value for a positive test was defined as 0.13 plus the mean OD of negative control wells. Specimens were considered positive for SARS-CoV antibodies if the calculated value (observed OD readings minus OD reading of the blank control) exceeded the cutoff value.

Laboratory D performed serologic testing for SARS with an in-house indirect EIA (2). Briefly, serially diluted serum specimens were added to 96-well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) precoated with either γ-irradiated SARS-CoV lysate (Urbani strain) or Vero E6 cell lysate and incubated at 37°C for 1 h. After being rinsed with PBS-Tween-20, plates were incubated with goat anti-human immunoglobulin (Ig) G, IgA, and IgM conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) at 37°C for 1 h. After washing, 2,2-azo-bis(3-ethylbenzthiazolin sulfonic acid) (ABTS) substrate (Kirkegaard and Perry Laboratories, Inc.) was added for 30 min at 37°C. ODs were measured at 410 nm and 490 nm wavelength. Specimens were considered positive for SARS-CoV antibodies if the adjusted sum OD (sum of differences between SARS-CoV antigen and control antigen wells) for

Table 1. WHO network laboratories that tested specimens from the four SARS patients in Guangdong Province, China[a]

	SARS-CoV						OC43/229E			
Laboratory code[b]	Real-time RT-PCR	RT-PCR Sequencing	EIA	IFA	Neutralization	Isolation	RT-PCR	EIA	IFA	Neutralization
A	√	√	√	√		√				
B	√	√	√		√	√				
C	√	√	√	√	√	√			√	
D	√	√	√		√	√	√	√		√

[a]WHO, World Health Organization; SARS, severe acute respiratory syndrome; CoV, coronavirus; RT-PCR, reverse transcription–polymerase chain reaction; EIA, enzyme immunoassay; IFA, immunofluorescence assay.
[b]Laboratory A, Center for Disease Control and Prevention of Guangdong Province and Center for Disease Control and Prevention of Guangzhou; laboratory B, Institute for Viral Disease Control and Prevention and Institute for Communicable Infectious Disease, Chinese Center for Disease Control and Prevention; laboratory C, Government Virus Unit and Department of Microbiology, Queen Mary Hospital; laboratory D, U.S. Centers for Disease Control and Prevention.

the 1:100 through 1:6,400 dilutions exceeded 1.25 and the titer of the specimen was ≥1:400. The titer for a specimen was taken as the highest dilution that had a positive adjusted OD value >0.21.

SARS-CoV Immunofluorescence Assay

Laboratory A performed an in-house immunofluorescence assay (IFA). Briefly, SARS-CoV (strain F69)–infected Vero E6 cells spotted and acetone-fixed on glass slides were prepared in advance of testing. Serum samples with a starting dilution of 1:25 were deposited onto the slides and incubated for 30 min at 37°C. The slides were then rinsed and blot-dried, and a fluorescein-labeled polyvalent anti-human immunoglobulin (Biosource International, Camarillo, CA) was added and incubated for 30 min at 37°C. The slides were then rinsed twice, mounted with phosphate-buffered glycerol and coverslip, and examined with a UV microscope. The immunofluorescence titer was taken as the highest dilution that showed a positive reaction (apple green fluorescent cytoplasmic granules). Laboratory C performed a similar IFA but modified it to use SARS-CoV (strain 6109)–infected FRhK4 cells.

229E/OC43 Microneutralization Assay

A microneutralization assay for human coronaviruses 229E and OC43 was developed for this study by laboratory D. Rhabdomyosarcoma (RD) cells were grown to 80%–90% confluence in Dulbecco's Modified Eagle's medium (DMEM) (GIBCO-Invitrogen, Carlsbad, CA) containing 10% defined fetal bovine serum (FBS) (HyClone, Logan, UT). 229E (ATCC VR-740) and OC43 (ATCC VR-759) were introduced onto the washed monolayers, and cultures were incubated at 33°C with 6% CO_2 for 5 days. The infected cultures were submitted to three freeze-and-thaw cycles, clarified by low-speed centrifugation, and stored at –70°C. Virus stocks were titrated by adding 50 µL of 10-fold dilutions (eight replicates per dilution) of 229E and OC43 to 96-well culture plates (Costar, Cambridge, MA), followed by adding 50 µL of 2 x 10^5 RD cells/mL and incubating at 33°C for 5 days. Infected wells were identified by an immunofluorescence assay as described elsewhere (13) by using monoclonal antibodies specific to 229E or OC43 nucleoprotein. The 50% tissue-culture infective dose ($TCID_{50}$) was determined by the method of Reed and Muench (14). For the microneutralization assay, 50 µL of heat-inactivated human and animal control hyperimmune serum samples were serially twofold diluted in 10% FBS-DMEM in triplicate wells. Each sample was diluted in duplicate 96-well tissue culture plates followed by adding 100 $TCID_{50}$ of 229E or OC43. After 1 h incubation, 50 µL of 2 x 10^5 RD cells/mL was added, and plates were incubated at 33°C in 6% CO_2 for 5 days. A back titration was included in each test. Neutralization titers were defined as the reciprocal of the highest serum dilution that completely inhibited fluorescence in at least two of the three triplicate wells.

OC43 Immunofluorescence Assay

Laboratory C performed an in-house IFA, as described above for SARS-CoV, but it was modified to use OC43-infected BSC-1 cells.

OC43/229E Enzyme Immunoassay

Laboratory D performed serologic testing for human coronaviruses by using an in-house indirect EIA. Briefly, 96-well microtiter plates (Dynatech Laboratories) were coated overnight at 4°C with previously optimized concentrations of clarified lysates of OC43 and 229E and uninfected RD cells as prepared for the microneutralization assay above. Serially diluted serum specimens (1:100 through 1:3,200) were added and incubated for 1.5 h at 37°C. After being washed with PBS-Tween-20, the plates were incubated at 37°C for 1 h with goat anti-human IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO). After similar washing, a tetra-methyl benzidine substrate (Zymed Laboratories, San Francisco, CA) was added and incubated for 15 min at room temperature. The colorometric reaction was stopped with 2 mol/L phosphoric acid, and ODs were measured in dual wavelength mode at 450 nm and 650 nm. The adjusted sum OD of duplicate wells of the positive and negative antigen was determined for each serum dilution, and the highest dilution showing a

≥0.1 OD was taken as the virus-specific IgG antibody titer. A titer rise of more than fourfold between early and late serum pairs was considered evidence of recent infection with OC43 or 229E.

Molecular Studies

SARS-CoV Real-time RT-PCR

Respiratory specimens and stool were tested for SARS-CoV RNA by using real-time reverse transcription–polymerase chain reaction (RT-PCR) assays from several different sources. Laboratories A, B, and C used assays developed by Piji Bioengineering (Shenzhen, China) and Artus GmbH (Hamburg, Germany) following the manufacturers' instructions. Laboratory D used an in-house real-time RT-PCR procedure (15). Appropriate RT-PCR controls, including positive SARS RNA and negative extraction (water blank), and amplification controls were included in each run. In addition, laboratory D tested each specimen for human RNAse P to ensure the adequacy of RNA extraction and to monitor for RT-PCR inhibitors.

OC43/229E RT-PCR

RT-PCR for human OC43 and human 229E was performed on throat swabs by the degenerated consensus PCR primers for the genus *Coronavirus* by using the SuperScript One Step RT-PCR kit (Invitrogen). These primers were derived from a highly conserved region in open reading frame (ORF) 1b of the *pol* gene. Viral total nucleic acid was processed from throat swabs, and RT-PCR was carried out by using the method previously described (15).

SARS-CoV Sequencing

Sequences from the 3′ third of the SARS-CoV genome were obtained from overlapping RT-PCR products that covered the envelope (E), membrane (M), and nucleocapsid (N) structural protein genes, plus several other gaps of unknown function, such as S-E gap between S ORF and E ORF and M-N gap between S ORF and E ORF, by using a previously described method (16).

Culture

Virus isolation was attempted on RT-PCR–positive respiratory specimens collected from patients 1 and 2 by methods previously described (2). Briefly, 100 μL of antibiotic-treated specimen was introduced into tube cultures of Vero E6 cells and incubated at room temperature for 1 h. Fresh modified DMEM with 2% fetal calf serum was added, and cultures were incubated at 37°C with rocking. Cultures were observed daily for cytopathic effect for 2 weeks then blind passaged. Negative cultures for SARS-CoV were confirmed by RT-PCR as described.

Results

Serologic Testing

All but one of the serum specimens from these patients tested positive for SARS-CoV antibodies by all laboratories using multiple assay formats, including EIA, IFA, and neutralization assay (Table 2). All four patients had detectable SARS-CoV antibodies by one or more laboratories very early in the illness; serum specimens collected 6 days after onset from patients 1 and 2 were positive by all laboratories by one or more methods, and specimens collected at 8 days from patients 3 and 4 were positive by EIA performed at laboratories A and B, respectively. Where comparisons could be made, the pattern of antibody responses were similar for all assays, and a fourfold or greater rise in EIA or IFA antibodies was demonstrable in multiple laboratories in three of the four patients. A fourfold rise in SARS-CoV antibodies in patient 3 was identified by only one laboratory (laboratory A) by IFA; laboratory A was the only laboratory that tested the earliest specimen from patient 3 and tested the serum specimens as they arrived and not concurrently.

A concurrent rise in OC43 antibodies was detected by IFA (laboratory C) in patient 4. To assess the possibility of OC43-induced SARS antibodies reacting with SARS-CoV and confounding the diagnosis of SARS, early and late serum specimens from all patients were simultaneously tested by laboratory D for SARS-CoV, OC43, and 229E antibodies by neutralization assay and EIA (Table 3). In these tests, no rises in either EIA or neutralizing antibody titers were noted to OC43 or 229E. The serum pair from patient 1 had a rise in SARS neutralizing antibodies, and the serum pair from patient 2 had a rise in SARS EIA antibodies (Tables 2 and 3). The earliest acute-phase serum specimens for patients 2–4 were unavailable for these tests. Neutralizing antibody titers were not detected to 229E and were detected at a lower titer to OC43 than to SARS-CoV; previous studies have shown a lack of SARS-CoV antibodies in paired serum specimens from patients with acute 229E and OC43 infections (2).

Virus Detection

A variety of specimens were tested for SARS-CoV by culture isolation and for SARS-CoV RNA by multiple real-time RT-PCR assays (Table 4). Although virus was not successfully isolated from any of the respiratory specimens, viral RNA was detected by RT-PCR in several respiratory specimens from patients 1 and 2 by two or more laboratories and by one laboratory from a single stool specimen from patient 4. In contrast, all respiratory specimens were negative for other coronaviruses by RT-PCR. The RT-PCR–positive throat swabs were collected on days 6, 8, and 10 for patient 1 and on days 6 and 8 for patient 2.

Table 2. SARS-CoV EIA, IFA, and neutralization test results for the four SARS patients in Guangdong Province, China[a]

Patient	No. days[b]	EIA				IFA		Neutralization		
		A	B	C[c]	D	A	C	B	C[d]	D
1 (onset 12/16/2003)	6	4	160	+	400	10	25	16	10	20
	7	16	320	+	1,600	80	200			
	8	32	640			160				
	9	128	1,280							
	10	128	1,280			160				
	11	128	1,280	+	6,400	160	200			
	12	128	1,280					64	160	
	13	128	1,280							
	15	64		+	6,400		400			
	22	64			6,400					160
2 (onset 12/26/2003)	6		10			10		≤8	40	
	7	4	20	+		80	100			
	8	8	40		1,600	160				160
	9		80			160				
	10		80			160				
	11	16			6,400	160				
	17			+	6,400		1,600	16	160	
	19	16								
	21				6,400					160
	22	16								
3 (onset 12/30/2003)	8	16								
	9		160		6,400	20				160
	10	16	320	+		80	200		80	
	11	64	320			320				
	13	16		+	6,400		400	32	160	
	17	32			6,400					160
4 (onset 1/8/2004)	8	–	10				<25		<10	
	11		10							
	15	80	160		1,600	320	1,600			80
	17	160								
	18				1,600				20	
	21	160			1,600					80

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus; EIA, enzyme immunoassay; IFA, immunofluorescence assay; +, positive, –, negative. Where no value appears, the test was not performed.
[b]Number of days the specimen was collected after symptom onset.
[c]Specimens tested by Government Virus Unit, Hong Kong Special Administrative Region and Department of Microbiology, Queen Mary Hospital.
[d]Specimens tested by Government Virus Unit, Hong Kong Special Administrative Region, with a subset tested by Department of Microbiology, Queen Mary Hospital.

The amount of viral RNA in these specimens was small, as shown by threshold cycle values >35 with the real-time RT-PCR assays and inconsistent positivity between laboratories for all but the day 6 throat swab from patient 1. None of the other types of specimens, including multiple stool or stool swab specimens on three of the patients, tested positive for SARS-CoV RNA. Only the throat swab collected on day 6 from patient 1 had sufficient viral RNA for the initial sequencing studies. Sequences were confirmed to be SARS-CoV and most closely matched isolates from civet cats taken in November and December from wild animal markets in Guangdong Province (17). A more extensive description of these sequences will be presented in a follow-up report.

Discussion

The participation of multiple laboratories in this study documented SARS-CoV infection in these patients and permitted comparisons of results obtained with different assays. Antibody testing provided a relatively early indication of SARS-CoV infection in all four patients, as early as 6 days but no later than 9 days after onset of illness. SARS-CoV antibodies have been reportedly detected as early as 6 days after onset, but they are more commonly detected after 10 to 14 days (2,4,18). Although this early appearance of antibody is consistent with antibody response seen during the 2003 SARS outbreak, it could also indicate differences from that outbreak. For example, these patients may have had a longer incubation period or may have been previously infected with a SARS-like coronavirus that primed their immune systems for a rapid anamnestic antibody response. A longer incubation period, possibly because of low virus inoculum or infection with a virus that replicated less efficiently, could have provided addi-

Table 3. SARS-CoV, OC43, and 229E neutralization test results for the four SARS patients in Guangdong Province, China[a]

Patient	No. days[b]	SARS-CoV			OC43[c]	229E[c]
		B	C	D	D	D
1 (onset 12/16/2003)	6	16	10	20	20	<20
	12	64	160			
	22			160	20	<20
2 (onset 12/26/2003)	6	≤8	40			
	8			160	20	<20
	17	16	160			
	21			160	20	<20
3 (onset 12/30/2003)	9			160	40	<20
	10		80			
	13	32	160			
	17			160	40	<20
4 (onset 1/8/2004)	8		<10			
	15			80	20	<20
	18		20			
	21			80	20	<20

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus. When no values appear, the test was not performed.
[b]Number of days the specimen was collected after symptom onset.
[c]OC43 and 229E neutralization tests were conducted by laboratory D only.

tional time to mount an antibody response, leading to an apparent rapid antibody induction.

SARS-CoV was not isolated from any patient, and viral RNA was detected in only 3 of 4 patients at relatively low levels. This finding is consistent with results from studies during the 2003 outbreak that suggested high virus titers are associated with more severe illness and more efficient virus transmission (19,20). All of the patients survived, and none showed evidence of transmission to others. Only one stool specimen from one of these patients (patient 4), collected during week 2 of illness, was positive for SARS-CoV RNA. This finding contrasts with reports from the 2003 outbreak that a high percentage of stool specimens collected during week 2 of illness were positive for SARS-CoV by RT-PCR and that stool specimens were more likely to be positive than other specimens during week 2 of illness (4). The low virus titer found in these patients may reflect infection acquired directly from animals, before the virus acquired genetic changes that facilitate infection in humans. Sequence data recently reported by the Chinese SARS Molecular Epidemiology Consortium (21) suggest that SARS-CoV may have adapted to humans during the 2003 outbreak. Authors noted that the S protein gene had a higher ratio of coding to noncoding changes in the early stages of the outbreak, compared with later stages. This finding suggests a selective advantage for these coding changes (presumably related to infection in humans) and is consistent with findings from other coronaviruses that amino acid changes in the S protein can affect tissue tropism and disease associated with infection (22).

A previously unrecognized concern is the potential for serologic cross-reactions between human coronaviruses OC43 and 229E and the SARS-CoV. During the course of the workup of these patients, one laboratory showed an antibody response to both SARS-CoV and OC43 in one patient. This reaction was unexpected and required further testing to definitively determine which virus induced the antibody response. Subsequent neutralization and EIA antibody results demonstrate that SARS-CoV, not OC43- or 229E-like coronaviruses, induced the antibodies detected. The weight of the evidence therefore suggests that these patients were infected with SARS-CoV and not OC43- or 229E-like coronaviruses. This finding reinforces the need to better understand mechanisms underlying apparent cross-reactions between SARS-CoV and other human coronaviruses with nonneutralization serologic assays.

These cases illustrate the diagnostic difficulties that can occur in evaluating patients for SARS. The ability to confidently confirm or negate a diagnosis allows control efforts to focus on the most important cases and minimizes unnecessary social and economic disruption. The cost of missing a case can be high if further spread occurs, and the cost of false-positive diagnosis to the patient, family members, healthcare facility, and community can also be substantial.

In response, WHO established the SARS Reference and Verification Laboratory Network, which verifies all suspected cases of SARS-CoV infection outside the country in which the cases occur (11). WHO and other groups have also begun to provide test samples and proficiency panels that allow laboratories to assess their assays' performance and guidelines for specimen management to minimize the chance of contamination. The rapid identification and confirmation of SARS-CoV infection in these four cases exemplify the successful collaboration between local and

Table 4. SARS-CoV real-time RT-PCR test results for the four SARS patients in Guangdong Province, China[a]

Patient	No. days[b]	Specimen type	Real-time RT-PCR			
			A	B	C[c]	D
1 (onset 12/16/2003)	6	Throat swab	+	+	+	+
	7	Stool	–	–	–	–
		Sputum	–	–	–	–
	8	Throat swab	+	–	–	–
		Stool	–	–	–	–
		Serum	–	–	–	–
	9	Throat swab	–	–	–	–
		Stool	–	–	–	–
		Serum	–	–	–	–
		Sputum	–	–	–	–
		Urine	–	–	–	–
	10	Throat swab	+		–	
		Stool	–		–	
		Serum	–		–	
		Urine	–		–	
	11	Throat swab	–	–	–	–
		Stool	–	–	–	–
		Serum	–	–	–	–
		Sputum	–	–	–	–
		Urine	–	–	–	–
	12	Throat swab		–	–	–
		Stool		–	–	–
		Serum		–	–	–
		Urine		–	–	–
	13	Throat swab		–	–	–
		Stool		–	–	–
		Serum		–	–	–
		Urine		–	–	–
	14	Sputum			–	–
	15	Throat swab			–	–
		Stool			–	–
2 (onset 12/26/2003)	6	Throat swab	–	+		
	7	Throat swab	–	–		–
	8	Throat swab		+		+
	9	Throat swab		–		
	10	Throat swab	–	–		
3 (onset 12/30/2003)	9	Throat swab	–	–		–
		Stool	–	–		–
		Urine	–	–		–
	10	Throat swab	–			–
	11	Throat swab		–		
4 (onset 1/8/2004)	12	Stool	–	–	+	–
	15	Throat swab	–	–	–	–
	16	Stool	–		–	–

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus; RT-PCR, reverse transcription–polymerase chain reaction; +, positive, –, negative. Where no value appears, the test was not performed.
[b]Number of days the specimen was collected after symptom onset.
[c]Specimens tested by Government Virus Unit, Hong Kong Special Administrative Region, with a subset tested by Department of Microbiology, Queen Mary Hospital

WHO Network Laboratories and highlight the importance of continued cooperation in the event of the appearance of new SARS cases.

Dr. Liang is a professor and virologist specializing in arboviruses with a focus on Japanese encephalitis and alphavirus research.

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Egg Quality Assurance Programs and Egg-associated *Salmonella* Enteritidis Infections, United States

Gerald A. Mumma,* Patricia M. Griffin,* Martin I. Meltzer,* Chris R. Braden,* and Robert V. Tauxe*

A *Salmonella enterica* serovar Enteritidis epidemic in the United States began in 1978, spread to much of the country in the following decade, and began declining in 1996. We examined correlations between annual changes in *S.* Enteritidis incidence in humans and introductions of egg quality assurance programs (EQAPs) in some states to reduce *S.* Enteritidis contamination of eggs. Before EQAPs, 62% of the changes in *S.* Enteritidis incidence were higher than the baseline for each state. After EQAPs, 73%–84% of the changes were below the baseline. Regression analysis showed that a 1% increase in the number of eggs produced under an EQAP was associated with a 0.14% decrease in *S.* Enteritidis incidence ($p < 0.05$). These data indicate that EQAPs probably played a major role in reducing *S.* Enteritidis illness in these states.

An epidemic of infections caused by *Salmonella enterica* serovar Enteritidis in the United States began in New England in 1978 and spread to much of the rest of the country in the next decade. Though the spread has declined in all regions since 1996 (Figure 1), the number and incidence of *S.* Enteritidis infections have not shown substantial decline since 1999 (1). Since grade A shell eggs have been implicated as a major source of *S.* Enteritidis infections in humans in the United States (2), interventions have been introduced to reduce *S.* Enteritidis infection in poultry and eggs and the resulting illness in humans (3–10). These interventions include State Egg Quality Assurance Programs (EQAPs), which are voluntary programs that are based on Hazard Analysis Critical Control Point (HACCP) principles and designed around production, management, and monitoring practices to mitigate risk for *S.* Enteritidis contamination of eggs (3,11,12). Motivations for egg producers to adopt an EQAP may include scientific, public health, public relations, or marketing reasons (13). Initially, producers enrolled voluntarily into state- or industry-sponsored EQAPs. However, in some states, commercial egg producers are required to participate in EQAPs because egg processors, food commodity brokers, insurance companies, and integrated commercial companies are increasingly demanding producer participation in EQAPs as a condition of egg sales (12).

Research to date has focused on verifying the role and effectiveness of EQAPs in mitigating *S.* Enteritidis in layer flocks and eggs (4,5,13). Effectiveness might be indicated by reductions of *S.* Enteritidis prevalence in layer flocks (11,14,15), farm environments (11,16), and eggs produced by infected flocks (5) after introducing EQAPs. Reported reductions in *S.* Enteritidis rates in markets with EQAPs have been used to explain the effectiveness of EQAPS in reducing *S.* Enteritidis illness in humans (2,3,11,16,17). Some evidence shows that interventions that reduce the storage time of shell eggs, internal or ambient temperature, or prevalence of *S.* Enteritidis–positive flocks or that increase diversion of eggs from *S.* Enteritidis-positive flocks to pasteurization, may help reduce the incidence of *S.* Enteritidis in humans (18). However, little is known about the contribution of each intervention to the overall reduction in the number of *S.* Enteritidis cases. The present study analyzes flock-based EQAPs to assess their actual contribution to the reduction of *S.* Enteritidis incidence in humans.

Methods

Baseline incidence was defined as *S.* Enteritidis incidence in the year in which an EQAP was adopted in a state or group of states affected by the *S.* Enteritidis epidemic. We calculated *S.* Enteritidis incidence for a state or group

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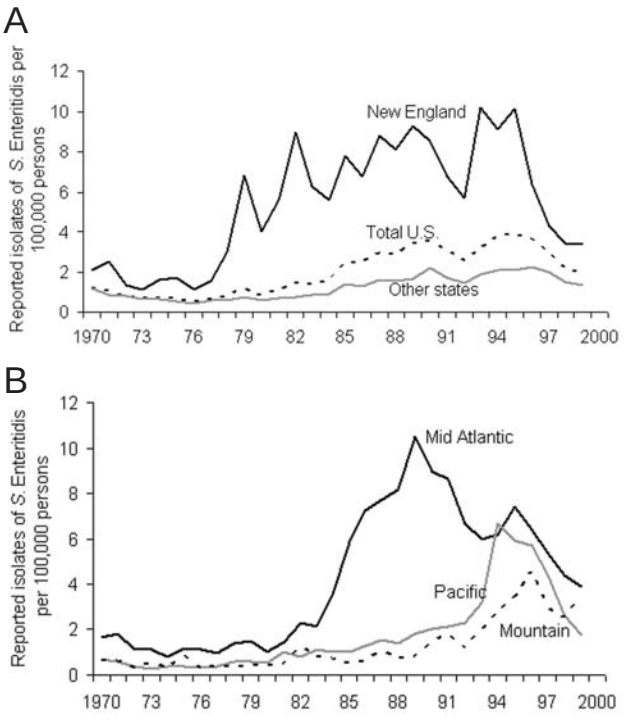

Figure 1. Reported isolates per 100,000 persons of *Salmonella enterica* serovar Enteritidis by region, United States, 1970–1999. (A) New England: Connecticut, Maine, New Hampshire, Rhode Island, Vermont. (B) Mid-Atlantic: New Jersey, New York, Pennsylvania. Pacific: Alaska, California, Hawaii, Oregon, Washington. Mountain: Arizona, Colorado, Montana, Nevada, New Mexico, Utah. Other states: Alabama, Arkansas, Delaware, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maryland, Michigan, Minnesota, Mississippi, Missouri, Nebraska, North Carolina, North Dakota, Ohio, Oklahoma, South Carolina, South Dakota, Tennessee, Texas, Virginia, Washington DC, West Virginia, Wisconsin. Source: Centers for Disease Control and Prevention, National Salmonella Surveillance System (1).

of states as the number of reported human *S.* Enteritidis isolates in a year divided by that state's or group of states' population for that year expressed per 100,000 persons. We defined a state affected by the *S.* Enteritidis epidemic as one for which the *S.* Enteritidis incidence was >1/100,000 in any year between 1980 and 1999.

States that adopted EQAPs were grouped into state- and industry-sponsored EQAPs. We grouped EQAPs into state-sponsored and industry-sponsored types on the basis of whether the state government was actively involved in third-party monitoring, supervision, provision of technical advice, and procedure of handling houses that are found to be *S.* Enteritidis positive. In this study, state-sponsored EQAPs were defined as having active state Department of Agriculture and Department of Health involvement in providing technical advice, supervising and monitoring the programs, requiring third-party auditing, testing eggs for contamination with *S.* Enteritidis if houses were positive, and diverting eggs found to be contaminated with *S.* Enteritidis to pasteurization and hard cooking. Industry-sponsored EQAPs were defined as lacking state government involvement, recommending but not requiring third-party audits of the program, and recommending immediate extra cleaning of *S.* Enteritidis–contaminated houses upon depopulation of the houses (19).

We calculated the percentage change in annual *S.* Enteritidis incidence relative to the baseline (hereafter referred to as the change in *S.* Enteritidis incidence):

$$(1)\ \Delta RI_{it} = \left[\frac{\left(I_{it} - I_{it_0}\right)}{I_{it_0}}\right],$$ where, I stands for SE incidence; ΔRI for change in SE incidence; $i = 1, 2, ..., N$ for state; $t = -T, ..., -1, 0, 1, ..., T$ for time, t_0 = year of EQAPs introduction

We then divided the change in *S.* Enteritidis incidence for a given year by the number of years before or after the intervention to get the annualized percentage change in *S.* Enteritidis incidence (hereafter referred to as the annualized change in *S.* Enteritidis incidence). We used two methods to examine changes in *S.* Enteritidis incidence: a simple change-point procedure and regression analysis.

Change-point Analysis Framework

We constructed a graph with a horizontal axis representing time in years and a vertical axis representing *S.* Enteritidis incidence (Figure 2A). If an intervention is effective, *S.* Enteritidis incidence should decrease after the baseline year (line b, Figure 2A). If the *S.* Enteritidis incidence had been increasing before the intervention, a smaller increase in incidence after the baseline would also show evidence of effectiveness (line c, Figure 2A). If the *S.* Enteritidis incidence had been decreasing before the intervention, we would expect a faster decrease in incidence after the baseline. Similarly, the lack of change in *S.* Enteritidis incidence observed before, during, and after the intervention would be evidence of lack of effect (line d, Figure 2A), and an increase in *S.* Enteritidis incidence (line e, Figure 2A) would be evidence that the intervention was associated with an acceleration of the epidemic.

The effectiveness of an intervention can further be assessed by using the annualized change in *S.* Enteritidis incidence (Figure 2B). To show evidence of an intervention's effectiveness, we would expect the annualized change in *S.* Enteritidis incidence after intervention to be below the baseline rate (dotted line b, Figure 2B) or the preintervention rate (dotted line b compared to a, Figure 2B). A more modest effect after intervention that shows the epidemic continuing to grow at a diminished annualized rate would find the change in incidence above baseline but below the preintervention level (dotted line c compared to a, Figure 2B). An ineffective intervention would result in an annualized change in *S.* Enteritidis incidence after intervention that is equal to the rate before

intervention (dotted line d compared to a, Figure 2B). An annualized change in *S.* Enteritidis incidence above the preintervention rate (line e compared to a, Figure 2B) would be evidence that the intervention was associated with an acceleration of the epidemic. If changes in *S.* Enteritidis incidence continued at about the same rate or were sustained for a number of years, the annualized change in *S.* Enteritidis incidence would, in time, trend toward the baseline. We calculated Yates corrected chi-square values to verify whether changes in observations of annualized *S.* Enteritidis incidence were statistically significant.

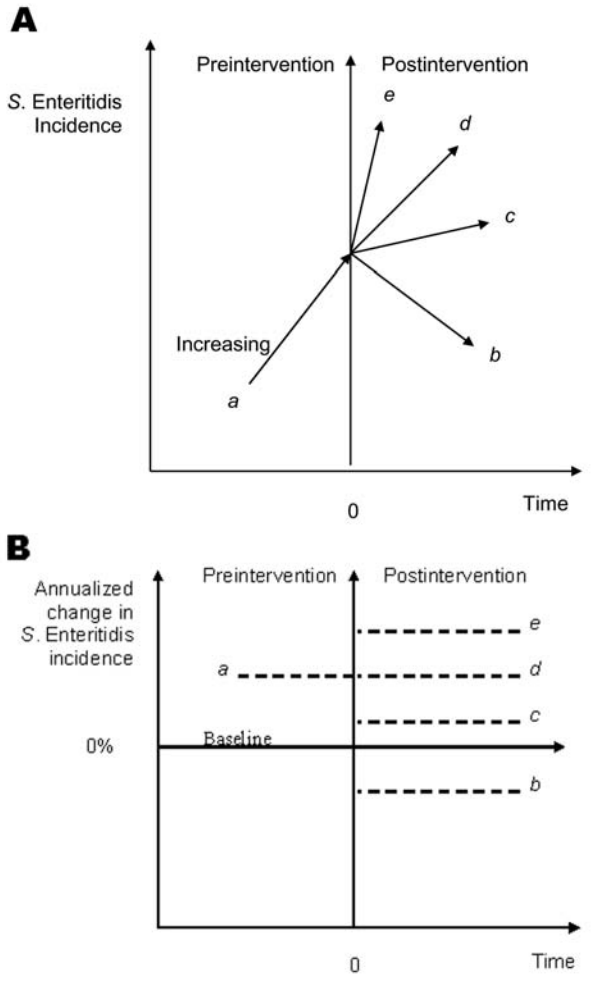

Figure 2. A) Framework to compare *Salmonella enterica* serovar Enteritidis incidence for a given year to the baseline incidence for evidence of intervention effectiveness. Each arrow shows the change of *S.* Enteritidis incidence for a given year, relative to baseline incidence. The letter *a* shows increasing *S.* Enteritidis incidence relative to the baseline incidence, *b* shows a reduction in *S.* Enteritidis incidence relative to the baseline incidence, *c* shows a smaller reduction, *d* shows no change, and *e* shows an increase. B) Framework to compare annualized changes in *S.* Enteritidis incidence for a given year to a baseline incidence for evidence of effectiveness of an intervention. Each dot represents an annualized change in *S.* Enteritidis incidence relative to the baseline change in *S.* Enteritidis incidence, which is 0%. The letter *a* shows preintervention annualized changes in *S.* Enteritidis incidence that are above the baseline, indicating annual increase in incidence; *b* shows postintervention annualized changes in *S.* Enteritidis incidence that are below the baseline; and preintervention rates indicating annual decrease in incidence; *c* are above baseline but below preintervention rates; *d* are above baseline but equal to preintervention rates; and *e* are above preintervention and baseline rates.

We examined observations of pre-EQAP annualized rates of change in *S.* Enteritidis incidence for a period of up to 5 years and for a variable period of up to 8 years of post-EQAP observations. The time periods selected were considered to be long enough to include any relevant lag effects and short enough to exclude confounding influence of other interventions, such as those that require refrigerating eggs. To analyze the timing of the decrease in *S.* Enteritidis incidence, we grouped states on the basis of duration of postintervention follow-up and then type of EQAP and compared the annualized changes in *S.* Enteritidis incidence for each of the 5 years before adopting EQAPs and up to 5 years after adopting EQAPs. For example, states that adopted EQAPs in 1996 had 3 years of common experience with the intervention from 1996 to 1999 and formed a group based on this common length of time. The annualized changes in *S.* Enteritidis provided a measure for comparing incidence of *S.* Enteritidis before, during, and after EQAP adoption.

Regression Model

We also examined the percentage change in *S.* Enteritidis incidence by using a pooled regression model. The pooling method can be used to combine cross-section and time-series data. This technique allows for the error terms to have equal variance on the chosen values of the explanatory variables within a state, but unequal variance between states (20–25), which results in efficient and unbiased parameter estimates. We estimated a pooled regression equation for five cross-sectional states (Connecticut, Louisiana, Indiana, Pennsylvania, and California) for 5 years post-EQAP by using SHAZAM (20). The pooled regression equation was:

(2) $Y_{it} = X_{it}\beta + \varepsilon_{it}$ where i = 1, 2..., N and stands for state; t = 1,..., T for time, Y for the percentage change in SE incidence; X for explanatory variables; β for *k*X1 column vector of the unknown parameters $\beta_1, \beta_2, ..., \beta_k$; $k = 1,2,..., K$ for number of unknown parameters; and ε_{it} for a random error term.

We included Louisiana in the regression model, although it was unaffected by the epidemic, to improve the degrees of freedom for the model. Only four of the states that were affected by the *S.* Enteritidis epidemic had 5 years of post-EQAP experience. Also, we included a dummy variable in the model to control for states that were not affected by the *S.* Enteritidis epidemic.

Independent variables (and types) were percentage of eggs produced in participating farms (continuous), type of EQAP (binary: state- or industry-sponsored: yes/no), num-

ber of United States Department of Agriculture (USDA) *S.* Enteritidis outbreak traceback investigations (continuous), proportion of population at high risk for *S.* Enteritidis (children <5 years and seniors >65 years) (continuous), and whether the state was in the northeast geographic region (Connecticut, Pennsylvania, New York) (binary: yes/no). The proportion of eggs produced under EQAPs by state and year was the index to measure participation in EQAPs.

Data Collection

We sent a detailed questionnaire to state veterinarians and public health officials in all states that were involved with *S.* Enteritidis control and prevention efforts. We also asked state officials to share the questionnaire widely with stakeholders (e.g., state departments of agriculture, laboratory workers, and egg industry officials) in state *S.* Enteritidis mitigation efforts.

The questionnaire collected data on whether egg producers in the state had adopted an EQAP, and if so, the type of EQAP to which most producers in the state adhered (e.g., industry-or state-sponsored), year of EQAP initiation, estimated proportion of total commercial layer-flock participation in the EQAP by year, and elements of the EQAP to which participants were required to adhere. The annual number of *S.* Enteritidis cases was obtained from reports by state and local health departments to the National *Salmonella* Surveillance System (1). Estimates of the annual population data for states were obtained from the Bureau of the Census of the U.S. Department of Commerce (26).

The annual numbers of eggs produced by state from 1972 to 1999 were obtained from USDA's National Agricultural Statistics Service (NASS) (27). To calculate the proportion of eggs produced under an EQAP for each state, we assumed no difference in egg production per layer between layers raised under an EQAP and those raised under no EQAP. We then calculated the proportion of eggs produced under each EQAP as a product of the proportion of total layer flocks that participated in the EQAP and the annual total number of eggs produced by each state. This calculation may overestimate the annual total number of eggs produced for human consumption. The category "table eggs" would provide a closer estimate of eggs produced for human consumption. However, due to confidentiality concerns, NASS does not publish complete information on table egg production.

To estimate a proxy for the proportion of the state's population at high risk for *S.* Enteritidis, we used the resident population <5 years of age and >65 years of age and total resident population. Data for estimates of the resident population by age and state for 1989–1999 were obtained from the U.S. Census Bureau (26). We obtained the number of successful *S.* Enteritidis outbreak traceback investigations (investigation to establish origin of *S.* Enteritidis–contaminated eggs) by state from USDA's *S.* Enteritidis Task Force Status Reports for 1990 to 1993 (8). Similar information was not available for tracebacks from 1996 to 1999, when the Food and Drug Administration was responsible for tracebacks. The typical procedure when a traceback leads to farms is for the regulatory body to take environmental samples of manure areas, egg belts and escalators, fans, and feed. If the environment tests positive for *S.* Enteritidis, the farmer can either divert the eggs to pasteurization or hard cooking for the lifetime of the flock, divert the eggs until they test negative for *S.* Enteritidis, or depopulate the flock.

Results

EQAP

Egg Production Under EQAP

We received analyzable results from 41 states. No response was received from Idaho, Maine, Mississippi, New Jersey, New Mexico, Virginia, Washington, Wisconsin, or West Virginia. These states accounted for ≈9% of U.S. shell egg production from 1989 to 1999. State officials in 15 of the 41 states reported that egg producers in their respective states had adopted one of two kinds of EQAPs from 1989 to 1999. Ten (Connecticut, Pennsylvania, California, South Carolina, Maryland, Ohio, Michigan, Utah, New York, Alabama) adopted state-sponsored EQAPs, and 5 (Louisiana, Indiana, Oregon, Florida, Georgia) adopted industry-sponsored EQAPs. Eleven of the 41 responding states were affected by the *S.* Enteritidis epidemic, of which 9 had state-sponsored programs and 2 had industry-sponsored programs. The proportion of eggs produced under EQAPs among the 41 responding states increased from 1% in 1989 to 46% in 1999, and eggs produced under EQAPs among the 11 states that had EQAPs and were affected by the *S.* Enteritidis epidemic increased from 3% in 1989 to 79% in 1999 (Table 1).

Change-point Analysis

We calculated 55 preintervention and 40 postintervention annualized changes in *S.* Enteritidis incidence for 11 states that were affected by the *S.* Enteritidis epidemic and adopted EQAPs (Table 2). Before adopting any EQAP (state- or industry-sponsored), *S.* Enteritidis incidence relative to the baseline was higher in 62% of the observations and lower in 38% of the observations (Figure 3). After EQAPs were introduced, *S.* Enteritidis incidence increased relative to the baseline in 28% of the post-EQAP observations and decreased in 73% of the observations, which indicates a significant reduction (Yates-corrected chi-square = 9.61, p = 0.0019).

Table 1. Eggs produced under EQAPs as a percentage of total egg production, 1989–1999[a]

Year	% of eggs produced under EQAPs	
	United States (N = 41)[b]	States with EQAPs affected by *S.* Enteritidis epidemic (N = 11)
1989	1.2	2.5
1990	1.7	2.8
1991	3.6	7.0
1992	5.2	10.5
1993	5.5	10.9
1994	8.6	18.0
1995	10.5	22.2
1996	17.6	38.3
1997	27.0	59.1
1998	34.7	68.8
1999	46.1	78.6

[a]EQAP, *Salmonella enterica* serovar Enteritidis egg quality assurance programs.
[b]Forty-one states responded to the survey. A state affected by the *S.* Enteritidis epidemic had an *S.* Enteritidis isolation rate >1/100,000 population in any year from 1980 to 1999. States with EQAPs affected by the *S.* Enteritidis epidemic were California, Connecticut, Indiana, Maryland, Michigan, New York, Ohio, Oregon, Pennsylvania, South Carolina, and Utah.

In the analysis restricted to the nine affected states that adopted state-sponsored EQAPs, we calculated 45 preintervention and 31 postintervention annualized changes (Table 2). *S.* Enteritidis incidence was higher than the baseline in 62% of the pre-EQAP observations and lower in 38% of the observations (Figure 4). After the state-sponsored EQAPs were introduced, *S.* Enteritidis incidence increased relative to the baseline in 16% of the observations and decreased in 84%, which indicates a significant reduction (Yates-corrected chi-square = 14.05, $p = 0.00018$).

To analyze the timing of reductions in *S.* Enteritidis incidence, we defined groups of 11, 7, 6, and 4 states with at least 1 year, 2 years, 3 years, and 5 years of post-EQAP follow-up, respectively. In each group, *S.* Enteritidis incidence was increasing before adoption of EQAPs and decreased afterwards. The effect of the intervention was apparent in the first year and was sustained (Figure 5).

Results of the Regression Model for States that Adopted EQAPs

Descriptive statistics for variables used in the regression model are presented in Table 3. A 1% increase in the quantity of eggs produced under an EQAP (state- or industry-sponsored) was associated with a 0.14% ($p < 0.05$) reduction in the change in *S.* Enteritidis incidence (Table 4). A state-sponsored EQAP was associated with a decrease of 72.25% ($p < 0.1$) in the change in *S.* Enteritidis incidence. A 1% increase in the population at high risk for *S.* Enteritidis was associated with an 8.15% ($p < 0.05$) increase of the change in *S.* Enteritidis incidence. An increase of 1 in the number of successful USDA *S.* Enteritidis outbreak traceback investigations was associated with an increase of 2.82% ($p < 0.001$) in the change in *S.* Enteritidis incidence. No significant associations were found for changes in *S.* Enteritidis incidence and states affected by the *S.* Enteritidis epidemic or located in the Northeast region.

Discussion and Conclusions

Although a decline in prevalence of *S.* Enteritidis in layer-flock eggs might indicate effectiveness of EQAPs in mitigating *S.* Enteritidis (4,6,10,12,16,28), a connection with reductions of *S.* Enteritidis infections in humans is necessary to indicate effectiveness of the programs in mitigating human illness. Our simple change-point procedure showed a connection between the introduction of EQAPs at the state level and significant reductions in *S.* Enteritidis incidence in humans. The regression analysis found that increasing the quantity of eggs produced under EQAPs was associated with reducing *S.* Enteritidis incidence.

Several factors limited this study. Whether an EQAP was introduced at the beginning of the year or at the end of the year might make a difference, and defining a baseline year might introduce error in the analysis. However, data about the month in which EQAPs were introduced were lacking for most states that adopted these programs. Although some EQAPs are similar in that they were designed through close collaboration among states, they vary in practice and motivation, which limits

Table 2. Annualized rates of change of *Salmonella enterica* serovar Enteritidis incidence before and after adoption of EQAPs in states affected by the epidemic[a,b]

S. Enteritidis incidence	With state- or industry-sponsored EQAPs (%)	With state-sponsored EQAPs (%)
Increasing before introduction of EQAPs	34 (62)	28 (62)
Decreasing before introduction of EQAPs	21 (38)	17 (38)
Increasing after introduction of EQAPs	11 (28)	5 (16)
Decreasing after introduction of EQAPs	29 (73)	26 (84)

[a]EQAP, egg quality assurance program. This analysis included 11 states that had state- or industry-sponsored EQAPs and 9 states with state-sponsored EQAPs. Departments of agriculture and health provided technical advice, supervision, and monitoring to state-sponsored EQAPs. State-sponsored required auditing by third parties, tested eggs for contamination with *S.* Enteritidis if layer houses were found to be positive, and diverted eggs found to be contaminated with *S.* Enteritidis to pasteurization and hard cooking.
[b]Percentages may not equal 100% because of rounding.

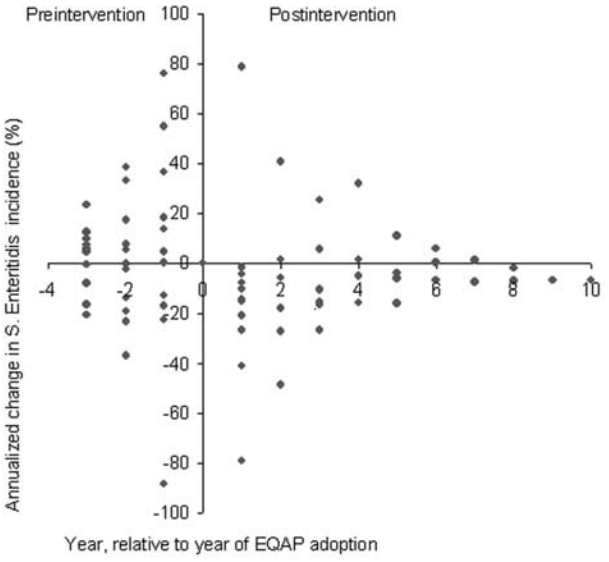

Figure 3. Observed annualized changes in *Salmonella enterica* serovar Enteritidis incidence for 11 states that were affected by the *S.* Enteritidis epidemic and adopted state- or industry sponsored EQAPS. The 11 states were California, Connecticut, Indiana, Maryland, Michigan, New York, Ohio, Oregon, Pennsylvania, South Carolina, and Utah.

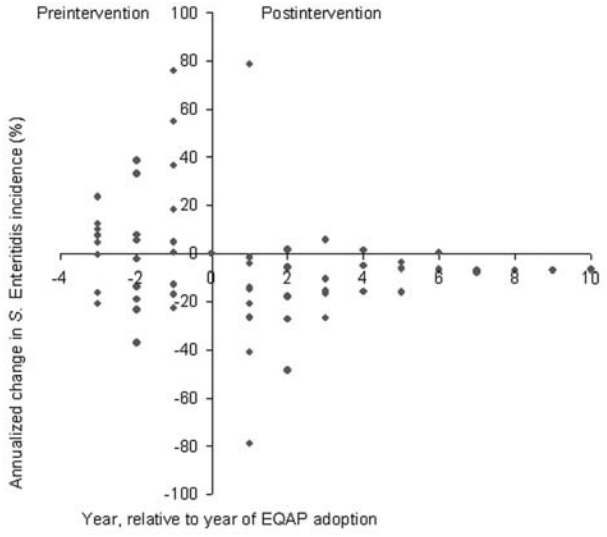

Figure 4. Observed annualized rates of change in *Salmonella enterica* serovar Enteritidis incidence for nine states that were affected by the *S.* Enteritidis epidemic and adopted state-sponsored EQAPS. The nine states were California, Connecticut, Maryland, Michigan, New York, Ohio, Pennsylvania, South Carolina, and Utah.

generalizations about all EQAPs, whether state or industry sponsored. We found verifying the exact practices of each EQAP to be difficult because EQAPs range from self-certification programs, like the 5-Star United Egg Producers program (19) practiced in Indiana and Oregon that does not require microbiologic testing for chicks, pullets, layers, and eggs, to the more structured, regulated, rigorous, and costly Pennsylvania Egg Quality Assurance Program (14). Eleven of 15 states with EQAPs reported that they required periodic sampling and testing of layer environments, layers, and eggs for *S.* Enteritidis, but 4 (Oregon, Louisiana, Indiana, Georgia) did not. All states that required microbiologic testing, except for Florida, involved their state governments in setting up and monitoring their EQAP programs.

We did not study interactions in the regression model because of few data points and degrees of freedom, which limited the robustness of its results. We were more interested in the direction (positive or negative) of the estimate of the percentage of eggs produced under EQAPs than the magnitude. Also, our results were based on unverified respondent estimates of the proportion of eggs produced under EQAPs, information about the type of EQAP, and when the EQAP was instituted. Further, because accurately estimating prevalence of diabetes, cancer, HIV/AIDS, and pregnancy at the state level was difficult, we used the population of children <5 years of age and seniors >65 years of age for each state to represent the population at high risk for *S.* Enteritidis.

We assumed that eggs produced in a state are applied to meet the consumption needs of that state, and changes in *S.* Enteritidis incidence within the state would reflect the

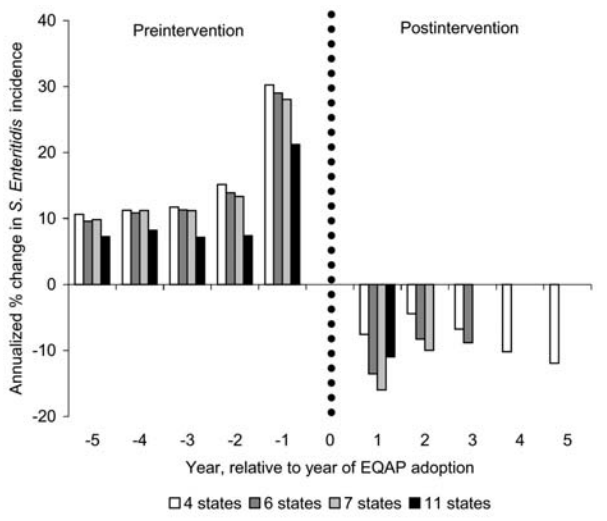

Figure 5. Annualized change in *Salmonella enterica* serovar Enteritidis incidence for groups of states that had egg quality assurance programs (EQAPs) for the same period within group and different periods among groups. The groups of states were 11 states with ≥1 year of post-EQAP follow-up (Connecticut, Indiana, Pennsylvania, California, South Carolina, Maryland, Ohio, Michigan, Utah, New York, Oregon), 7 states with ≥2 years of post-EQAP follow-up (Connecticut, Indiana, Pennsylvania, California, South Carolina, Maryland, Ohio), 6 states with ≥3 years of post-EQAP follow-up (Connecticut, Indiana, Pennsylvania, California, South Carolina, Maryland), and 4 states with ≥5 years of post-EQAP follow-up (Connecticut, Indiana, Pennsylvania, California).

Table 3. Descriptive statistics for variables in the regression model[a]

Variable	Average	SD
Rate of change in *Salmonella enterica* serovar Enteritidis incidence (%) after EQAP adoption	3.2	44.9
% eggs produced under EQAP by states	63.6	29.5
% a state's population at high risk for *S.* Enteritidis[b]	20.6	0.5
States with state-sponsored EQAP (%)[c]	60	50
States affected by the *S.* Enteritidis epidemic (%)	20	40
Successful *S.* Enteritidis outbreak traceback investigations by state per year[d]	0.3	1.1
States in the northeast region of United States (D)[e]	40	50

[a]EQAP, egg quality assurance program; D, dummy variable.
[b]Children <5 and seniors >65 as a proxy for population at high risk for *S.* Enteritidis.
[c]Not industry-sponsored EQAP.
[d]Number of *S.* Enteritidis outbreak investigations from United States Department of Agriculture *S.* Enteritidis Task Force Status Reports for 1990 to 1993.
[e]Northeast region includes Connecticut, Pennsylvania, and New York.

effect of the state's EQAP, but this assumption may not be accurate. Eggs in the United States are distributed widely across the nation through a dynamic system that makes it difficult to track the source and destination of eggs by state. Although data about the source and destination of eggs and egg products are desirable, they are not currently available (29).

Not all egg producers immediately join EQAPs, and the percentage of eggs produced in a state under an EQAP varies as producers adopt or leave EQAPs. The simple change-point analysis did not account for these variations and assumed that EQAPs were homogeneous within and among states. The regression model allowed EQAPs to be homogeneous within states and heterogeneous among states.

Our model could not estimate unreported cases in a meaningful way, although these cases constitute most cases of salmonellosis (14). The larger proportion of *S.* Enteritidis cases goes unreported (30). Other factors may have affected *S.* Enteritidis incidence in humans that we did not account for in this model because of lack of specific data, such as improvements in egg refrigeration during distribution and handling, traceback investigations from 1996 to 1999, and use of pasteurized eggs. However, these measures were not implemented in tandem with the EQAPs within or among states. Therefore, the close temporal association between implementing EQAPs and decreasing rates of *S.* Enteritidis infection indicate the importance of EQAPs as a control strategy.

The results of our study indicate that flock-based interventions have had a positive effect on health by reducing *S.* Enteritidis incidence in humans. These data further indicate that EQAPs probably played a major role in reducing *S.* Enteritidis illness in the United States. Considering that as of 1999, less than half of shell eggs in the United States were produced under EQAPs (Table 1), and that the number of cases and relative rate of *S.* Enteritidis have not shown significant decline since 1999, adopting EQAPS by producers and states would likely improve the public's health and prevent reemergence of egg-based *Salmonella*.

Acknowledgments

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Table 4. Regression model estimated rates of change in *Salmonella enterica* serovar Enteriditis incidence associated with unit changes in related variables[a]

Explanatory variable	Unit of change	Change in *S.* Enteritidis rate (%)	p value
Intercept[b]		–120.65	< 0.01
Eggs produced under EQAP (%)	1	–0.14	< 0.05
State population at high risk for *S.* Enteritidis (%)[c]	1	–8.15	< 0.01
State had a state-sponsored EQAP[d]	Yes	–72.25	< 0.1
State was affected by *S.* Enteritidis epidemic[e]	Yes	–3.60	
Successful *S.* Enteritidis outbreak traceback investigations by state per year[f]	Numeral	2.82	< 0.01
State was in the northeast region of the United States[g]	Yes	12.36	

[a]EQAP, egg quality assurance program.
[b]Intercept term is the baseline case, which represents no eggs produced under EQAPs, zero percent of the population at high risk for *S.* Enteritidis, no states affected by *S.* Enteritidis epidemic, no successful outbreak investigations, and all states outside of the northeast region.
[c]Children <5 and seniors >65 years.
[d]Not industry-sponsored EQAP.
[e]A state affected by *S.* Enteritidis epidemic had an isolation rate >1/100,000 persons from 1980 to 1999.
[f]Number of *S.* Enteritidis outbreak investigations from USDA *S.* Enteritidis Task Force status reports for 1990 to 1993.
[g]Northeast region includes Connecticut, Pennsylvania, and New York.

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Dengue Emergence and Adaptation to Peridomestic Mosquitoes

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Phylogenetic evidence suggests that endemic and epidemic dengue viruses (DENV), transmitted among humans by the anthropophilic mosquitoes *Aedes aegypti* and *Ae. albopictus*, emerged when ancestral, sylvatic DENV transmitted among nonhuman primates by sylvatic *Aedes* mosquitoes adapted to these peridomestic vectors. We tested this hypothesis by retrospectively examining evidence for adaptation of epidemic and endemic versus sylvatic strains of DENV-2 to *Ae. albopictus* and *Ae. aegypti*. First and second-generation offspring of mosquitoes from different geographic regions in the Americas and Southeast Asia were tested for their susceptibility to epidemic/endemic and sylvatic DENV-2 isolates from West Africa, Southeast Asia, and Oceania. Both *Aedes* species were highly susceptible (up to 100% infected) to endemic/epidemic DENV-2 strains after ingesting artificial blood meals but significantly less susceptible (as low as 0%) to sylvatic DENV-2 strains. Our findings support the hypothesis that adaptation to peridomestic mosquito vectors mediated dengue emergence from sylvatic progenitor viruses.

Dengue is caused by any of four antigenically distinct serotypes of dengue virus (DENV), family *Flaviviridae*. An estimated 100 million annual dengue cases occur each year in tropical cities, in which more than 2.5 billion people (almost half of the global population) are at risk (1). Infection with one DENV serotype confers lifelong protection against homologous reinfection, while a subsequent heterologous infection increases the likelihood of a more severe form of the disease (2–4).

Dengue has four clinical manifestations: 1) undifferentiated illness, 2) classic dengue fever, 3) dengue hemorrhagic fever, and 4) dengue shock syndrome. Undifferentiated dengue, the most common syndrome, occurs when a DENV infection is asymptomatic or mildly symptomatic. Dengue fever involves an abrupt febrile illness lasting 2–7 days, accompanied by malaise, headache, retroorbital pain, myalgia, and arthralgia of such great intensity that it has earned the lexicon "break-bone" fever (5,6). Dengue hemorrhagic fever progresses to hemorrhagic manifestations and plasma leakage caused by increased vascular permeability. Dengue shock syndrome is characterized by circulatory failure and is the most lethal dengue syndrome (7).

Within forest habitats of West Africa, Malaysia, and probably Vietnam, zoonotic, sylvatic dengue cycles have been described involving *Aedes* spp. mosquitoes and monkeys (8–10). Sylvatic DENV vectors in Africa include *Aedes (Stegomyia) africanus*, *Ae. (S.) luteocephalus*, *Ae. (S.) opok*, *Ae. (Diceromyia) taylori*, and *Ae. (D.) furcifer* (10); in Malaysia, *Ae. niveus* has been implicated in transmission (8,9). These sylvatic cycles, probably involving only DENV-2 in West Africa but all four serotypes in Malaysia, are believed to represent the ancestral DENV cycles from which epidemic/endemic (henceforth referred to as endemic) strains of DENV-1–4 evolved independently hundreds to thousands of years ago (11). Although humans occasionally become infected with sylvatic DENV in West Africa and perhaps in Asia, they are tangential to the maintenance cycle, which involves sylvatic *Aedes* spp. mosquito vectors and nonhuman primates as reservoir hosts. In contrast to the sylvatic cycles, epidemic DENV cycles involving transmission among humans by *Ae. (Stegomyia) aegypti*, *Ae. (S.) albopictus*, and other anthropophilic *Aedes* species have emerged in large tropical

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urban cities (12). These urban cycles are ecologically and evolutionarily independent of the ancestral sylvatic cycles, with humans serving as reservoir hosts.

Dengue is a reemerging disease in the neotropics and is transmitted primarily by *Ae. aegypti*. The abundance in Africa of closely related *Aedes* species within the *Stegomyia* subgenus, the lack of closely related *Stegomyia* species in the Americas, and the existence of sylvatic *Ae. aegypti* (*Ae. aegypti formosus*) in Africa suggest an African origin for this species (13–17). Movement of people and their requisite water storage containers during the 17th to 19th centuries probably spread *Ae. aegypti* throughout the tropics and subtropics. After World War II, *Ae. aegypti* prevalence and distribution increased in Asia and the Pacific Islands. *Ae. aegypti* was partially eradicated from tropical America in the 1940s and 1950s, but peridomestic *Ae. aegypti aegypti* has now reinfested most of the neotropics (12).

The Asian tiger mosquito, *Ae. albopictus*, originally of sylvatic origin as well, has spread widely in the world since the 1970s, including to the United States, Latin America, tropical Africa, the Pacific Islands, and Europe (7). Although less anthropophagic than *Ae. aegypti*, it is a secondary vector of DENV and possibly of greater importance in the early historical stages of urban dengue emergence.

We hypothesized that all four endemic dengue viruses evolved independently from sylvatic progenitors by adaptating to peridomestic mosquito vectors and human reservoir hosts (11). The rise of urban civilizations and the associated peridomestication of *Ae. aegypti* and *Ae. albopictus* mosquitoes provided this opportunity for adaptation and resulted in the emergence of dengue in urban areas of the tropics. This hypothesis predicts that endemic DENV strains are more efficient at infecting urban mosquitoes such as *Ae. aegypti* and *Ae. albopictus* than are the ancestral, sylvatic DENV strains. We tested this hypothesis by using experimental infections of *Ae. aegypti* and *Ae. albopictus* with sylvatic versus urban strains of DENV-2. Our results support the hypothesis that adaptation to peridomestic mosquito vectors mediated dengue emergence from sylvatic progenitor viruses.

Methods

Mosquito Colonies

Because geographic variation exists with regard to susceptibility to DENV in both colonized (18,19) and wild-collected populations of *Ae. aegypti* and *Ae. albopictus* (20–22), mosquitoes from the United States, Brazil, Bolivia, and Thailand were tested. These locations were selected to represent a wide geographic range, including regions with endemic dengue, and on the basis of availability of specimens from collaborators. Because laboratory colonization has been shown to affect susceptibility of mosquitoes to oral infection by flaviviruses (23,24), low filial generation cohorts were used for susceptibility experiments. *Ae. aegypti* and *Ae. albopictus* females were collected during the fall of 2001 from Galveston, Texas, and the first filial (F1) laboratory generation was used for experiments. F1 generation *Ae. aegypti* females were also hatched from eggs collected in Mae Sed, Tak, Thailand, in 2002. Second generation (F2) *Ae. aegypti* collected in Santa Cruz, Bolivia, in 2002 were also used. From Brazil, F1 *Ae. albopictus* from Pindamonhangaba City (an urban environment) and F1 and F2 *Ae. albopictus* from Pedrinhas City (a rural environ) were used from a parental collection in 2001. All mosquitoes were maintained in an insectary at 28°C, with a relative humidity of 80% and a 12:12 light-dark circadian cycle. Adults were fed a hamster blood meal to obtain eggs. Eggs were stored in plastic containers for up to 3 months. Larvae were reared on a diet of ground rabbit and mouse chow. Pupae were transferred to screened cages, and adults were fed 10% sucrose ad libitum.

Virus Strains

Low-passage isolates of DENV-2 were selected for this study to represent similar geographic ranges for endemic and sylvatic strains and based on the ability to obtain high-titered stocks after passage in mosquito cell (C6/36) cultures (Table 1); the strains included endemic strains New Guinea C (prototype strain) and 1349 and sylvatic strains PM 33974, A2022, and P81407. Virus stocks were prepared on C6/36 cell cultures and quantified by infecting C6/36 cells in 96-well plates with serial dilutions, followed by cell

Table 1. DENV-2 strains used in this study[a]

Virus strain	Virus type	Host	Passage history[b]	Blood meal titer ($log_{10}TCID_{50}$/mL)	Location	Year
1349	Endemic	Human	Mosquito 2, C6/36 2	6.5	Burkina Faso (Upper Volta)	1982
New Guinea C	Endemic	Human	Monkey 1, mosquito 4, C6/36 1	8	New Guinea	1944
PM33974	Sylvatic	*Aedes africanus*	*Toxorhynchites amboinensis* 1, C6/36 2	8	Guinea	1981
DAK AR 2022	Sylvatic	*Ae. africanus*	SM6, C6/36-2	10	Burkina Faso (Upper Volta)	1980
P8-1407	Sylvatic	Sentinel monkey	SM3, C6/36-2	9.5	Malaysia	1970

[a]DENV, dengue virus; $TCID_{50}$, 50% tissue culture infective dose; SM, suckling mouse.
[b]C6/36, *Ae. albopictus* cell culture.

spotting in 12-well slides and immunofluorescence assays (IFA) to determine 50% tissue culture infective doses ($TCID_{50}$) (see below). All work with DENV was carried out in a biosafety level 2 laboratory at the University of Texas Medical Branch with recommended safety procedures (25).

Indirect Fluorescent Antibody Test

Infection of mosquitoes with DENV was assayed with IFA in C6/36 cells, which is more sensitive than direct IFA of mosquito tissues (data not shown). Mosquito bodies and legs were triturated in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, glutamine, and antimicrobial agents (penicillin and streptomycin). Ten microliters of each triturated suspension was added to 90 µL of MEM in 96-well microtiter plates. Plates were incubated for 7 days at 28°C. After incubation, 10 µL suspensions of C6/36 cells were placed on multiwell slides, air dried, and fixed in ice-cold 80% acetone. Slides were then incubated for 1 h at 37°C with a polyclonal anti-DENV-2 mouse ascitic fluid diluted 1:80 in phosphate-buffered saline (PBS). Slides were rinsed twice in PBS and overlaid with fluorescein isothiocyanate–labeled goat anti-mouse immunoglobulin G (Sigma, St. Louis, MO) diluted 1:15 in PBS. Slides were again incubated for 1 h at 37°C and washed twice in PBS. Slides were examined at 200 to 1,000x with an inverted fluorescent microscope.

Vector Susceptibility

Artificial blood meals consisting of 1% sucrose, 20% fetal bovine serum, 5 mmol ATP, 33% PBS-washed sheep blood cells, and 33% MEM were used for mosquito susceptibility determinations. Multiple cohorts of 30 to 50 mosquitoes were offered blood meals incubated at 37°C in a water-jacketed membrane feeder (23). After 1 h of feeding, engorged mosquitoes were sorted from unengorged ones, and a sample of the blood meal was assayed to determine the virus titer. Fully engorged mosquitoes were incubated for 14 days at 27°C with a 12:12 light-dark cycle. Then, legs were detached from cold-anesthetized mosquitoes and assayed to determine the dissemination rate of the virus from the midgut into the hemocoel (mosquito legs include hemolymph, which is believed to mediate infection of the salivary glands). Bodies were assayed to determine the overall infection rate. Blood meal titers were determined by IFAs on C6/36 cells (see above) of samples collected immediately after mosquito feeding.

Because mosquito infections caused by artificial blood meals are inefficient compared to those using viremic hosts, we used the highest virus titers available (6.5–10.0 $\log_{10}$ $TCID_{50}$/mL) from cell culture passages for our experiments. To interpret our data as conservatively as possible, infection and dissemination rates for endemic strains were only compared with rates from the same or higher blood meal titers for sylvatic strains. Infection and dissemination differences were tested for significance with chi-square and Fisher exact tests with the SPSS (Chicago, IL) Base 11.5 statistical package. When no significant differences were observed among endemic or sylvatic strains tested, data were pooled for each group.

Results

Aedes aegypti Susceptibility

After ingestion of artificial blood meals containing 6.5–8.0 $\log_{10}$ $TCID_{50}$/mL of endemic DENV-2, infection and dissemination rates in *Ae. aegypti* mosquitoes from Galveston, Texas, were 86.5%–100.0% and 90.6%–78.9%, respectively (Table 2). Infection and dissemination rates after exposure to sylvatic viruses were more variable but lower, ranging from 11.4% to 69.4% and from 0% to 64.4% after ingestion of 8.0 to 10.0 $\log_{10}$ $TCID_{50}$/mL of DENV. Even after the (lowest) infection rate data for strain 1407 were removed from the pooled analysis because they were significantly different than those for the other sylvatic strains, both infection ($p < 0.0001$) and dissemination ($p = 0.01$) rates were different between endemic and sylvatic strains (Table 2).

Ae. aegypti from Santa Cruz, Bolivia, had lower infection and dissemination rates than Galveston populations after being exposed to endemic DENV strains. Infection rates were 40.9%–48.1% and dissemination rates were 76.9%–77.8% with blood meal titers of 9.5 $\log_{10}$ $TCID_{50}$/mL (Table 3). Infection and dissemination rates in Bolivian mosquitoes exposed to sylvatic viruses were also lower than those of *Ae. aegypti* from Galveston, and sylvanic strain infection rates in Bolivian mosquitoes were lower ($p = 0.015$), ranging from 16.7% to 27.3%, than those of endemic strains; dissemination rates were not significantly different ($p = 0.663$).

Ae. aegypti from Mae Sed, Tak, Thailand, were also

Table 2. DENV-2 infection and dissemination rates in *Aedes aegypti*, Galveston[a,b]

Dengue strain	% infected (totals)	% dissemination[c] (totals)
1349 (endemic)	86.5 (32/37)	90.6 (29/32)
New Guinea C (endemic)	100 (38/38)	78.9 (30/38)
33974 (sylvatic)	54.2 (26/48)	61.5 (16/26)
2022 (sylvatic)	69.4 (25/36)	64 (16/25)
1407 (sylvatic)	11.4 (4/35)	0 (0/4)
Collapsed[d]		
Endemic	93.3 (70/75)	84.3 (59/70)
Sylvatic	63.0 (51/81)	62.7 (32/51)

[a]DENV, dengue virus.
[b]Blood meal titers are found in Table 1.
[c]Number of infected mosquitoes with virus in the legs.
[d]Strain 1407 data were not included in the collapsed analysis because they were significantly different from data for other sylvatic strains.

Table 3. DENV-2 infection and dissemination rates in *Aedes aegypti*, Bolivia[a,b]

Dengue strain	% infected (totals)	% dissemination[c] (totals)
1349 (endemic)	48.1 (13/27)	76.9 (10/13)
New Guinea C (endemic)	40.9 (27/66)	77.8 (21/27)
33974 (sylvatic)	16.7 (4/24)	100 (4/4)
2022 (sylvatic)	27.3 (6/22)	83.3 (5/6)
Collapsed		
Endemic	43 (40/93)	77.5 (31/40)
Sylvatic	21.7 (10/46)	90 (9/10)

[a]DENV, dengue virus.
[b]Blood meal titers are found in Table 1.
[c]Number of infected mosquitoes with virus in the legs.

less susceptible than the Galveston population to DENV-2 strains used in this study, with the exception of endemic DENV-2 strain 1349 from Burkina Faso (infection and dissemination rates were 94.3% and 80%, respectively, with this strain) (Table 4). Like the Galveston and Bolivian populations, the Thai population exhibited consistent differences in susceptibility to endemic versus sylvatic strains (33.0%–94.3% infection with the endemic strains vs. 0%–13% for sylvatic strains; 84.8%–90.9% dissemination rate for endemic strains vs. 0%–50% for sylvatic strains). Infection rates for both endemic strains were higher than for the pooled sylvatic rates ($p < 0.001$), while dissemination rates were not significantly different ($p > 0.1$).

Ae. albopictus Susceptibility

Like *Ae. aegypti*, *Ae. albopictus* from Galveston, Texas, exhibited greater susceptibility to endemic than sylvatic DENV strains. After ingesting blood meals containing 6.5–8.0 $\log_{10}$ $TCID_{50}$/mL of endemic strains, 92.3%–100% of mosquitoes became infected, with high rates of dissemination (Table 5). In contrast, only 11.1% of mosquitoes became infected after ingesting 8.0 $\log_{10}$ $TCID_{50}$/mL of the sylvatic strain. The infection rates for endemic strains were higher than for sylvatic strains ($p < 0.0001$), but the difference in dissemination was not significant ($p = 1.000$; only one infected mosquito with a sylvatic strain was tested and exhibited dissemination).

Table 4. DENV-2 infection and dissemination rates and *Aedes aegypti*, Thailand[a,b]

Dengue strain	% infected (totals)	% dissemination[c] (totals)
1349 (endemic)	94.3 (33/35)	84.8 (28/33)
New Guinea C (endemic)	33 (11/33)	90.9 (10/11)
33974 (sylvatic)	13 (6/46)	50 (3/6)
2022 (sylvatic)	8.1 (3/37)	0 (0/3)
1407 (sylvatic)	0 (0/27)	0 (0/0)
Collapsed[d]		
Sylvatic	8.2 (9/110)	33.3 (3/9)

[a]DENV, dengue virus.
[b]Blood meal titers are found in Table 1.
[c]Number of infected mosquitoes with virus in the legs.
[d]Data for the two endemic strains were not collapsed because they were significantly different.

Ae. albopictus from two different locations in Brazil were also tested. One location was urban, Pindamonhangaba, while the other, Pedrinhas, was rural. Again, a significant difference in susceptibility was observed between endemic (strain New Guinea C) and sylvatic (strain 33974) infections with F1 mosquitoes of both geographic locations (Pindamonhangaba, $p < 0.001$, Pedrinhas, $p < 0.001$). However, no significant differences were detected in dissemination rates for either F1 mosquito population ($p > 0.1$). For the Pedrinhas mosquito population, F2 mosquitoes were tested with additional endemic and sylvatic strains. Again, the endemic strains infected at a higher rate ($p < 0.001$) than sylvatic strains, even when blood meal titers were lower (Table 6). Dissemination rates were also markedly different ($p = 0.004$).

Table 5. DENV-2 infection and dissemination rates in *Aedes albopictus*, Galveston[a,b]

Dengue strain	% infected (totals)	% dissemination[c] (totals)
1349 (endemic)	100 (12/12)	91.6 (11/12)
New Guinea C (endemic)	92.3 (12/13)	75 (9/12)
33974 (sylvatic)	11.1 (1/9)	100 (1/1)
Collapsed		
Endemic	96 (24/25)	83.3 (20/24)
Sylvatic	11.1 (1/9)	100 (1/1)

[a]DENV, dengue virus.
[b]Blood meal titers are found in Table 1.
[c]Number of infected mosquitoes with virus in the legs.

Geographic Variation in Susceptibility among Mosquito Populations

Geographic variation for DENV susceptibility has been reported previously for both *Ae. aegypti* and *Ae. albopictus* (18,19). We found geographic variation among populations of both species. In general, *Ae. aegypti* from Galveston, Texas, were more susceptible than those from Bolivia ($p < 0.001$) but not those from Thailand ($p > 0.1$). *Ae. albopictus* from Galveston were also more susceptible to DENV-2 infection than those collected in Brazil ($p = 0.009$).

Overall Trends

In general, in the 701 peridomestic mosquitoes from four localities used in this study, we found high susceptibility to endemic DENV-2 isolates but much less susceptibility to sylvatic strains. These differences were detected despite the blood meal titers of sylvatic strains being equal to or greater than those of endemic strains. Dissemination rates within infected mosquitoes generally showed no significant difference between endemic and sylvatic strains. Our data also indicated that *Ae. albopictus* was more susceptible to endemic DENV-2 strains than *Ae. aegypti*

Table 6. DENV-2 infection and dissemination rates in *Aedes albopictus* (Brazil)[a,b]

Geographic population, generation	Dengue strain	% infected (totals)	% dissemination[c] (totals)
Pindamonhangaba F1			
	New Guinea C (endemic)	76.9 (10/13)	90 (9/10)
	33974 (sylvatic)	10.7 (3/28)	100 (3/3)
Pedrinhas F1			
	New Guinea C (endemic)	100 (10/10)	100 (10/10)
	33974 (sylvatic)	10 (2/20)	50 (1/2)
Pedrinhas F2			
	1349 (endemic)	100 (17/17)	88 (15/17)
	New Guinea C (endemic)	95.7 (22/23)	100 (22/22)
	33974 (sylvatic)	46.2 (6/13)	100 (6/6)
	2022 (sylvatic)	0 (0/15)	0 (0/0)
	1407 (sylvatic)	33.3 (5/15)	0 (0/5)
Collapsed (Pedrinhas F2)[d]			
	Endemic	49.3 (39/79)	94.9 (37/39)
	Sylvatic	21.4 (6/28)	100 (6/6)

[a]DENV, dengue virus; F2, second generation.
[b]Blood meal titers are found in Table 1.
[c]Number of infected mosquitoes with virus in the legs.
[d]Strain 2022 data were not included in the collapsed analysis because they were significantly different than data for the other sylvatic strains.

(overall 94% vs. 69% infection rates). Comparing *Ae. aegypti* and *Ae. albopictus* from one geographic location, Galveston, we did not find a difference between mosquito species when we compared infection with endemic strains 1349 ($p > 0.1$) or the New Guinea C strain ($p > 0.1$). However, Galveston *Ae. aegypti* were more susceptible to sylvatic strain 33974 than were *Ae. albopictus* from Galveston ($p = 0.026$).

Discussion

Historical Emergence of Dengue and Adaptation to Peridomestic Vectors

Our findings support the hypothesis that endemic DENV-2 strains are more efficient than sylvatic strains at infecting the peridomestic DENV vectors *Ae. aegypti* and *Ae. albopictus*. The overall trend that endemic DENV-2 strains were consistently more efficient at infecting peridomestic *Aedes* mosquitoes than were sylvatic DENV-2 strains ($p = 0.000$) supports our central hypothesis. Our data and previous phylogenetic studies (11) suggest that the emergence of endemic DENV from sylvatic progenitor strains occurred in conjunction with the peridomestication of *Aedes* mosquitoes and virus adaptation to these anthropophilic vectors. Although we tested only DENV-2 strains, emergence of DENV serotypes 1, 3, and 4 may also have been mediated by vector switching (from infecting sylvatic *Aedes* mosquitoes to *Ae. aegypti* and *Ae. albopictus*). Very few sylvatic DENV-1 and 4 strains are available (and none of DENV-3), which makes evaluating this hypothesis difficult.

The four independent evolutionary DENV emergence events (DENV-1–4) suggest that adaptation of DENV to new vectors and hosts occurred repeatedly from 300 to 1,500 years ago in Asia or Oceania (11,26). Since *Ae. aegypti* is not thought to have inhabited these regions at that time, *Ae. albopictus* was probably the original human vector (12). The widespread importance of *Ae. aegypti* as a vector may have begun in the 1700s, as commercial and slave trade transported it from its African origin. DENV-2 was probably introduced into Africa from Asia-Oceania approximately 1,000 years ago (11). The hypothesis that *Ae. albopictus* was the original peridomestic vector was supported by our study; *Ae. albopictus* was more susceptible to endemic DENV-2 strains than *Ae. aegypti*. The greater overall susceptibility (regardless of geographic origin) of *Ae. albopictus* compared to *Ae. aegypti* (94% and 69%, respectively) suggests a higher degree of adaptation, representing longer historical contact with *Ae. albopictus*. Other studies with sympatric populations from Brazil show *Ae. aegypti* to be more susceptible than *Ae. albopictus* to endemic DENV-2 (19,20).

Risk for Dengue in the United States

When the vectorial capacity of a mosquito for an arbovirus is considered, many factors come into play, including mosquito survivorship, density, proportion of infected mosquitoes that are feeding, extrinsic incubation period, vector susceptibility, and density of susceptible hosts (27). We used vector susceptibility in this study as a measure not only of epidemiologic importance but also of the extent of adaptation of a virus to its vector. However, the full competence of a vector is established not only by its ability to become infected but also by its ability to transmit a pathogen. This feature is what gives vector competence its epidemiologic importance. In our study, transmission potential was estimated from dissemination rates because previous studies have suggested that mosquitoes are capa-

ble of transmitting DENV as long as the virus is able to disseminate from the midgut into the hemocoel (i.e., there is no evidence of a salivary gland infection barrier) (18). Mosquitoes that have a disseminated infection were therefore assumed to be capable of transmission.

Current methods of dengue control rely primarily on mosquito control and are aimed at reducing the populations of urban vectors, especially *Ae. aegypti*. This mosquito was eradicated from much of the New World during the middle of the 20th century. After the termination of the *Ae. aegypti* eradication program, *Ae. aegypti* populations reinfested many of the New World countries from which they had been eliminated, probably from those that did not achieve eradication. Being well adapted to urban environments and competent for transmission, DENV has become the most important mosquitoborne virus in the neotropics. Air travel and migration have increased the movement of virus strains around the world. Dengue virus has frequently been imported into the United States, where local transmission has been reported (28). Much of the southern United States is at risk for dengue transmission because of the presence of endemic *Ae. aegypti* and *Ae. albopictus*. Our study suggests that local populations of both species from Galveston are highly susceptible and potentially able to transmit DENV-2 from Africa, Asia, and Oceania.

Implications for Dengue Control

Promising candidate dengue vaccines are raising hopes of effectively preventing human disease (29). Because humans are the only reservoir host for the endemic cycle, an effective vaccine could ultimately eradicate endemic strains. This scenario underscores the need for greater understanding of the historical emergence of human dengue from sylvatic origins to predict the facility with which the sylvatic strains could reemerge to initiate urban transmission. The four independent emergence events (DENV-1–4) suggest that the host-range changes that accompanied emergence can be readily accomplished by DENV; however, this hypothesis needs to be tested experimentally. One question to be answered is how many mutations are responsible for the efficient infection phenotype for *Ae. aegypti* and *Ae. albopictus* exhibited by the endemic DENV-2 strains. Identifying genetic determinants of DENV adaptation to these peridomestic vectors will ultimately provide an indication of the ability of these arboviruses to reemerge.

The viral molecular determinants that confer DENV with the ability to infect and be transmitted by their mosquito vectors are not known. Phylogenetic studies suggest that the DENV E protein may be important in the adaptation to urban vectors (11). In particular, domain III of the E protein contains several hypothetical amino acid replacements associated with emergence of urban strains. This clustering of changes in domain III is observed repeatedly during the emergence of DENV-1, DENV-2, and DENV-4, when phylogenetic methods are used. The envelope glycoproteins of other mosquitoborne viruses, including Sindbis (30), Venezuelan equine encephalitis (31–33), and La Crosse viruses (34), have been shown to mediate vector infection. Another genomic region potentially important in mediating vector transmission may be the 5′ noncoding region. Deletions in this region of DENV-4 constrain its ability to infect *Ae. aegypti* and *Ae. albopictus* mosquitoes (35).

Our study examined the extent of endemic DENV adaptation to peridomestic vectors. If this adaptation is species-specific, then sylvatic vectors may be more susceptible to infection by sylvatic than endemic DENV strains. We are currently evaluating this hypothesis with sylvatic West African vectors.

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Escherichia coli and Community-acquired Gastroenteritis, Melbourne, Australia

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As part of a study to determine the effects of water filtration on the incidence of community-acquired gastroenteritis in Melbourne, Australia, we examined fecal samples from patients with gastroenteritis and asymptomatic persons for diarrheagenic strains of *Escherichia coli*. Atypical strains of enteropathogenic *E. coli* (EPEC) were the most frequently identified pathogens of all bacterial, viral, and parasitic agents in patients with gastroenteritis. Moreover, atypical EPEC were more common in patients with gastroenteritis (89 [12.8%] of 696) than in asymptomatic persons (11 [2.3%] of 489, $p < 0.0001$). Twenty-two random isolates of atypical EPEC that were characterized further showed marked heterogeneity in terms of serotype, genetic subtype, and carriage of virulence-associated determinants. Apart from the surface protein, intimin, no virulence determinant or phenotype was uniformly present in atypical EPEC strains. This study shows that atypical EPEC are an important cause of gastroenteritis in Melbourne.

Strains of *Escherichia coli* that cause diarrhea are classified into pathotypes (or virotypes) according to their specific virulence determinants (1). These virulence determinants give each pathotype the capacity to cause a clinical syndrome with distinctive epidemiologic and pathologic characteristics. For example, enterohemorrhagic *E. coli* (EHEC) may cause hemorrhagic colitis and the hemolytic uremic syndrome because of their production of Shiga toxins, whereas enteroaggregative *E. coli* (EAEC) are associated with persistent diarrhea in children in less-developed countries (1). Enteropathogenic *E. coli* (EPEC) share several key virulence determinants with the most common varieties of EHEC, but lack Shiga toxins, and cause nonspecific diarrhea in infants in less-developed countries (2,3). EPEC also differ from EHEC in that they typically carry an EPEC adherence factor plasmid (EAF). This plasmid encodes both bundle-forming pili (Bfp) that promote bacterial adherence to mammalian cells and are required for virulence (4) and a transcriptional activator, known as Per, that upregulates genes, such as *eae*, within a pathogenicity island termed the locus for enterocyte effacement (LEE) (5). LEE is required to produce attaching-effacing lesions, which are characteristic of EPEC-induced pathology. A subset of EPEC, known as atypical EPEC, does not carry EAF and hence does not produce Bfp (3). The role of EPEC in disease is uncertain.

The principal reservoir of EHEC is food animals, in particular, cattle, which harbor these bacteria in the distal intestinal tract and from which bacteria can spread to humans through fecally contaminated food or water (1). Although the other pathotypes of diarrheagenic *E. coli* generally do not originate in animals, they may also spread to humans through food or water contaminated with excrement. Recently, we conducted a study to determine if the water supply of Melbourne, Australia's second largest city with >3 million inhabitants, is a source of intestinal pathogens that are responsible for community-acquired gastroenteritis. Among the pathogens that were sought were diarrheagenic *E. coli*, including atypical EPEC, which emerged as the predominant cause of gastroenteritis in this community.

Materials and Methods

The design of the Water Quality Study (WQS), which was conducted from September 1997 to February 1999, has been reported previously (6). Briefly, 600 Melbourne

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families, with at least two children 1–15 years of age, were enrolled in the study. Each family was allocated at random to receive a real or sham water treatment unit, which was installed in the kitchen of their home and supplied water through a separate faucet. Family members, comprising 2,811 persons, were followed for 15 months (68 weeks). Each participating household had a nominated member who completed a weekly questionnaire regarding the presence, duration, and severity of gastrointestinal symptoms. The primary endpoint of the study was highly credible gastroenteritis, which was defined as exhibiting any of the following symptoms in a 24-hour period: two or more loose stools, two or more episodes of vomiting, one loose stool together with abdominal pain or nausea or vomiting, or one episode of vomiting with abdominal pain or nausea. Cases of highly credible gastroenteritis were deemed to be distinct if the participant was symptom-free for at least 6 days.

Sample Collection and Processing

Participants in the study were asked to collect fecal specimens during episodes of gastroenteritis. A total of 795 specimens collected during 2,669 reported episodes of gastroenteritis were examined for rotavirus, adenovirus, Norwalk-like viruses, *Giardia* spp., and *Cryptosporidium* spp. and were cultured for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Vibrio* spp., *Yersinia* spp., *Aeromonas* spp., *Plesiomonas* spp., and *Clostridium difficile*, as described previously (6,7). Baseline frequencies of these pathogens in the study population were determined during the 4-month period, May through August 1997, immediately preceding the WQS. Frequencies were examined by investigating 1,091 fecal specimens from a convenience sample of participants. Participants who provided a baseline specimen were similar to those who did not provide a specimen in age, sex, and family background.

Examination of Feces for *E. coli*

Sufficient funds were available to investigate 1,250 samples for diarrheagenic *E. coli*. Of these samples, 500 were randomly selected from 1,091 fecal samples obtained from healthy persons in the baseline study, and 750 samples were randomly selected from the 795 samples obtained from participants with highly credible gastroenteritis in the WQS.

Bacteria were isolated from fecal samples by direct plating on MacConkey agar (Oxoid Ltd., Basingstoke, UK). After overnight incubation at 37°C, a sterile cotton swab was used to transfer the entire growth from each plate into Luria broth containing 30% (vol/vol) glycerol, which was then frozen at –70°C until required. Diarrheagenic strains of *E. coli* were identified by polymerase chain reaction (PCR) and confirmed by Southern hybridization. Template DNA for use in PCR was prepared from bacteria grown in 2.5 mL of MacConkey broth that contained a loopful of stored frozen culture and was incubated with shaking at 37°C overnight. One milliliter of this culture was centrifuged to pellet the bacteria, the supernatant was removed, and then the pellet was washed in 1 mL of phosphate buffer, resuspended in 200 μL sterile distilled water, and heated for 10 min at 100°C. Samples were then placed on ice for 5 min and recentrifuged at 16,000 x *g*. Aliquots of the supernatant were pipetted into sterile tubes and stored for <1 week at –20°C before use.

PCR amplifications were performed in a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) with AmpliTaq Gold polymerase (Applied Biosystems) and the primers listed in Table 1 in a reaction volume of 20 μL (for single reactions) or 50 μL (for multiplex PCR). The genes identified by these primers and their association with each pathotype of diarrheagenic *E. coli* are listed in Tables 1 and 2. PCR for the *lacZ* gene, which is found in almost all wild-type strains of *E. coli*, was included as a control to ensure that negative PCR assays were not a result of the absence of viable bacteria in the sample or the presence of inhibitors in the reaction mixture. Samples that were negative in the PCR for *lacZ* (3.6% of all those examined) were excluded from further analysis. At the conclusion of the PCR, 10 μL of the reaction mixture underwent electrophoresis on 2.5% 96-well format agarose gels (Electro-fast, ABgene, Epsom, UK). Gels were stained with ethidium bromide, visualized on a UV transilluminator, and photographed. A portion of the PCR product was retained for Southern blotting, which was performed by using capillary transfer of separated DNA fragments onto positively charged nylon membranes (Roche Diagnostics Ltd., Lewes, UK). Digoxigenin-labeled DNA probes were prepared by PCR (Roche Diagnostics) from the control strains (Table 2) by using the PCR primers listed in Table 1. The integrity of the probes was determined by nucleotide sequencing. Probes were hybridized overnight under conditions of high stringency at 65°C and detected by using chemiluminescence as recommended by the manufacturer. Probe-positive bacteria were assigned to a pathotype according the criteria in Table 2. Equivocal or ambiguous assays were repeated, and if results were still unclear, these specimens were excluded from further analysis.

Characterization of Isolates

Twenty-two EPEC isolates (11 from healthy persons and 11 from persons with diarrhea) were isolated in pure culture. Representative colonies of each were then serotyped with hyperimmune rabbit antisera (16). These strains were also subjected to PCR with the primers and conditions listed in Table 3 to determine intimin subtype

Table 1. Characteristics of the polymerase chain reaction (PCR) primers used to detect pathogenic *Escherichia coli* in mixed culture

Target gene or virulence factor	Primer[a]	Primer sequence (5′ to 3′)	PCR program (30 cycles)[b]	Product size (bp)	Reference
eae	1[1]	GACCCGGCACAAGCATAAGC	95°C, 30 s; 54°C, 90 s; 72°C, 90 s	384	(8)
	2[1]	CCACCTGCAGCAACAAGAGG			
ehxA	1[1]	GCATCATCAAGCGTACGTTCC		534	(8)
	2[1]	AATGAGCCAAGCTGGTTAAGCT			
stx1	1[2]	ATAAATCGCCATTCGTTGACTAC	95°C, 30 s; 52°C, 60 s; 72°C, 60 s[c]	180	(8)
	2[2]	AGAACGCCCACTGAGATCATC			
stx2	1[2]	GGCACTGTCTGAAACTGCTCC		255	(8)
	2[2]	TCGCCAGTTATCTGACATTCTG			
ltA	1[3]	GGCGACAGATTATACCGTGC	94°C, 60 s; 50°C, 60 s; 72°C, 120 s[c]	696	(9)
	2[3]	CCGAATTCTGTTATATATGTC			
st1A	1[3]	TCTGTATTATCTTTCCCCTC		186	(9)
	2[3]	ATAACATCCAGCACAGGC			
ipaC	1	CAGCAGATTGCAGCGCATAT	94°C, 30 s; 59°C, 90 s; 72°C, 90 s	811	This study
	2	CAAGAGCAGATGCATAACGC			
bfpA	1	ATTGAATCTGCAATGGTGC	95°C, 40 s; 55°C, 40 s; 72°C, 40 s	461	This study
	2	ATAGCAGTCGATTTAGCAGCC			
pCVD432	1	CTGGCGAAAGACTGTATCAT	94°C, 40 s; 53°C, 60 s; 72°C, 60 s	630	(10)
	2	CAATGTATAGAAATCCGCTGTT			
aggA	1	ATGCATTACTTTGGGTTTAG	94°C, 60 s; 50°C, 60 s; 72°C, 120 s[c]	414	This study
	2	TCAACCTTGACACTTGCC			
lacZ	1	TGATTGAAGCAGAAGCCTGC	94°C, 30 s; 59°C, 90 s; 72°C, 90 s	1,350	This study
	2	CGCCAATCCACATCTGTGAA			

[a]Primers with the same superscript were used in duplex PCRs.
[b]Before the first cycle, the sample was denatured for 10 min at 95°C; after the last cycle, the sample was extended for 8 min at 72°C.
[c]35 cycles.

and to investigate the presence of selected virulence-associated genes. The same 22 strains were also examined for their ability to adhere to and invade HEp-2 epithelial cells.

Bacterial Adhesion and Invasion of HEp-2 Cells

The Center for Vaccine Development (University of Maryland, Baltimore, MD) method was used to determine the pattern of bacterial adherence to HEp-2 epithelial cells (12). Bacterial strains were designated nonadherent if <10 of 200 HEp-2 cells had five or more bacteria attached. The fluorescent actin staining (FAS) assay, which correlates with the ability of *E. coli* to produce attaching-effacing lesions in the intestine, was performed by using a 6-h incubation period (22). At the completion of the assay, cells were examined by fluorescence and phase-contrast microscopy to confirm that fluorescent areas corresponded to attached bacteria. Bacterial strains that gave equivocal or negative results in the FAS assay were investigated for DNA corresponding to regions of the LEE, other than *eae*,

Table 2. Classification of pathogenic *Escherichia coli* according to amplicon(s) generated by polymerase chain reaction for virulence-associated determinants

	Gene or virulence-associated determinant[a]											
Interpretation[b]	pCVD432	*aggA*	*bfpA*	*eae*	*exhA*	*ipaC*	*stlA*	*ltA*	*stx1*	*stx2*	Control strain	Reference
EAEC[c]	+	+	–	–	–	–	–	–	–	–	O42	(11)
Typical EPEC	–	–	+	+	–	–	–	–	–	–	E2348/69	(12)
Atypical EPEC	–	–	–	+	–	–	–	–	–	–	E128012	(12)
EIEC	–	–	–	–	–	+	–	–	–	–	223/83	(13)
ETEC[c]	–	–	–	–	–	–	+	+	–	–	H10407	(14)
EHEC[d]	–	–	–	+	+	–	–	–	+	+	EDL933	(15)
STEC, not EHEC[e]	–	–	–	–	–	–	–	–	+	+		

[a]Factors specified by virulence genes: *aggA*, aggregative fimbria, AAF/I; *bfpA*, bundle-forming pilus; *eae*, intimin; *ipaC*, invasion plasmid antigen; *stlA*, heat-stable enterotoxin; *ltA*, heat-labile enterotoxin; *stx*, Shiga toxin.
[b]EAEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin–producing *E. coli*; EHEC, enterohemorrhagic *E. coli*.
[c]Either *stlA* or *ltA* positive.
[d]Either *eae* or *ehxA* and *stx1* or *stx2* positive.
[e]Either *stx1* or *stx2* positive.

Table 3. Characteristics of the polymerase chain reaction (PCR) primers used in this study for strain characterization

Primer	Primer sequence (5′ to 3′)	Target	PCR program (30 cycles)[a]	Product size (bp)	Reference
P1[b]	CTGAACGGCGATTACGCGAA				
P2	CCAGACGATACGATCCAG	*eae*[c]	94°C, 30 s; 53°C, 30 s; 72°C, 60 s	917	(17)
P3	CTGGAGTTGTCGATGTT	*eae*-α	94°C, 30 s; 53°C, 30 s; 72°C, 120 s	1,648	(17)
P4	GTAATTGTGGCACTCC	*eae*-β	94°C, 30 s; 53°C, 30 s; 72°C, 120 s	1,926	(17)
P5	GCCTCTGACATTGTTAC	*eae*-γ	94°C, 30 s; 53°C, 30 s; 72°C, 120 s	1,770	(17)
ecsD-lower	TATTTTCAAAAAGAATGATGTC	*eae*	94°C, 30 s; 56°C, 60 s; 72°C, 150 s	≈2,990[d]	(18)
SK1[e]	CCCGAATTCGGCACAAGCATAAGC				
LP5	AGCTCACTCGTAGATGACGGCAAGCG	*eae*-ε	94°C, 30 s; 55°C, 60 s; 72°C, 120 s	2,608	(18)
LP6B	TAGTTGTACTCCCCTTATCC	*eae*-ζ	94°C, 30 s; 53°C, 60 s; 72°C, 150 s	2,430	(18)
LP7	TTTATCCTGCTCCGTTTGCT	*eae*-ι	94°C, 30 s; 52°C, 60 s; 72°C, 150 s	2,685	(18)
LP8	TAGATGACGGTAAGCGAC	*eae*-η	94°C, 30 s; 52°C, 60 s; 72°C, 150 s	2,590	(18)
LP10	GGCATTGTTATCTGTTGTCT	*eae*-κ	94°C, 30 s; 52°C, 60 s; 72°C, 150 s	2,769	(18)
LP11B	GTTGATAACTCCTGATATTTTA	*eae*-θ	94°C, 30 s; 50°C, 60 s; 72°C, 150 s	2,686	(18)
LPFDF LPFDR	GAACTGTAGATGGGTAC AGCAGGCATAACGCAAG	*lpfD*	94°C, 60 s; 48°C, 50 s; 72°C, 60 s	798	(19)
Donne-280 Donne-281	CGGAACAGTAGGTTCACCTTC AGTGCCCGTGTTCTTGAACTG	*efa1*	94°C, 30 s; 50°C, 30 s; 72°C, 120 s	2,226	(20)
EASTOS1 EASTOS2	GCCATCAACACAGTATATCCG CGCGAGTGACGGCTTTGTAG	*astA*	94°C, 30 s; 50°C, 60 s; 72°C, 90 s	109	(10)
AggRks1 AggRkas2	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	*aggR*	94°C, 30 s; 50°C, 60 s; 72°C, 45 s[f]	254	(21)

[a]Before the first cycle, the sample was denatured for 10 min at 95°C; after the last cycle, the sample was extended for 7 min at 72°C.
[b]Primer P1 was used as forward primer in all PCR reactions in combination with P2, P3, P4, and escD-lower.
[c]Conserved region of *eae*.
[d]Amplicon sequenced.
[e]Primer SK1 was used as forward primer in all PCR reactions in combination with LP5, LP6B, LP7, LP8, LP10, and LP11B.
[f]35 cycles.

by colony blotting using the LEE-A, LEE-B, and LEE-D DNA probes and hybridization conditions described previously (23). Quantitative assessment of the ability of *E. coli* to invade HEp-2 cells was performed with the gentamicin-protection assay (24). Results were expressed as the number of bacteria recovered from HEp-2 cells after treatment with gentamicin as a percentage of the total number of cell-associated bacteria (24).

Statistical analysis was performed with InStat version 3.0 (GraphPad Software Inc., San Diego, CA). A p value < 0.05 was considered significant.

Results

Association of *E. coli* Pathotypes with Gastroenteritis

After excluding samples for which patient data were incomplete (12 samples), which were negative by PCR for *lacZ* (45 samples), or which gave equivocal results in the PCR or DNA hybridization assays (8 samples), 1,185 (94.8%) samples of the original 1,250 were available for analysis: 696 from patients with gastroenteritis and 489 from healthy persons. The results of the assays are summarized in Table 4. Enterotoxigenic (ETEC) and enteroinvasive (EIEC) strains of *E. coli* and Bfp-positive EPEC were identified in <0.5% of healthy persons or patients with gastroenteritis. EHEC were present in 4 (0.6%) of 696 of samples from persons with gastroenteritis and in no healthy persons, but the difference between the two groups was not significant (p = 0.15, Fisher exact test, 2-tailed). In contrast, both EAEC and atypical (Bfp-negative) EPEC were identified in >5% of patients, and the difference between the symptomatic and baseline groups was significant for each of these pathotypes (Table 4). Analysis of the data pertaining to patients in whom EAEC and atypical EPEC were identified showed that atypical EPEC were more frequent in younger persons; patients with atypical EPEC were a median age of 3.4 years, compared with 7.4 years for the symptomatic group overall (p < 0.0001; Mann-Whitney test, 2-tailed). Of all atypical EPEC in study participants with gastroenteritis, 75 (84%) were identified in children <10 years old, compared with 14 (16%) in patients ≥10 years (relative rate [RR] 3.4, 95%

Table 4. Frequency of *Escherichia coli* pathotypes in study participants with and without gastroenteritis

E. coli pathotype[a]	Source		p[b]
	Symptomatic (n = 696) (%)	Symptom-free (n = 489) (%)	
EAEC	45 (6.5)	15 (3.1)	0.02
Typical EPEC	2 (0.3)	1 (0.2)	NS
Atypical EPEC	89 (12.8)	11 (2.3)	< 0.0001
EIEC	0	0	NS
ETEC	2 (0.3)	0	NS
EHEC	4 (0.6)	0	NS
STEC, not EHEC	0	1 (0.2)	NS

[a]EAEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*; EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin–producing *E. coli*; EHEC, enterohemorrhagic *E. coli*; NS, not significant ($p > 0.1$).
[b]Fisher exact test, 2-tailed.

confidence interval [CI] 2.0–5.9). In contrast, the median age of patients infected with EAEC was 7.3 years.

Examining the seasonal occurrence of gastroenteritis from all causes showed that gastroenteritis was more common in the warmer months; 65.7% of cases occurred in the 6 months from October through March (Figure 1). Infections with EAEC and atypical EPEC reflected this distribution with 80% and 65.5% of infections with these bacteria, respectively, occurring during the same period. The high incidence of EAEC in February and March may have been caused by small family outbreaks; 9 of 25 cases during this period originated in three households.

In view of the seasonal variation in the occurrence of EAEC and atypical EPEC, we reanalyzed the data and compared the symptomatic and baseline groups, because the EPEC were examined only during the 4-month period from May through August, 1997. This analysis showed that the occurrence of EAEC in persons with gastroenteritis (6 [3.8%] of 157) and in asymptomatic persons (15 [3.1%] of 489) was essentially the same during May through August of 1998 and 1997, respectively (RR 1.2, 95% CI 0.5–3.0). In contrast, atypical EPEC were more frequent in the gastroenteritis group during the same period; 19 (12.1%) of 157 of symptomatic patients were positive for these bacteria, compared with 11 (2.3%) of 489 for the asymptomatic group (RR 5.7, 95% CI 3.1–10.5, $p < 0.0001$; Fisher exact test, 2-tailed). The frequency of atypical EPEC in symptomatic patients from households with real and sham water treatment units was similar (42 [12.3%] of 341 and 47 [13.2%] of 355, respectively).

Characterization of EPEC Isolates

Pure cultures of PCR- and probe-positive atypical EPEC were obtained from 22 randomly selected samples that were positive in the original PCR. These isolates were characterized further to establish their identity as EPEC and to determine if they belonged to a limited number of clones. All strains were identified as atypical EPEC in that they were negative for *bfpA*, *stx1*, *stx2*, *aggA*, and *aggR*. Determination of O:H serotype and intimin subtype revealed that although strains from each sample were the same, those obtained from different persons were highly heterogeneous, and no two isolates belonged to the same serotype and intimin subtype (Table 5). Only three isolates were of serotypes (O55:H7 and O126:H6 [two isolates]) that commonly include atypical EPEC. In addition, one isolate, W145, was serotype O55:H6, which includes typical EPEC strains (2). Eight isolates were O-serogroups that were classified as nontypeable because they did not react with any of the available O-typing sera (O1–O181), and one isolate had an H antigen that did not react with any of the available H-typing sera (H1–H56) and was classified as nontypeable.

Atypical EPEC strains were also heterogeneous in their carriage of putative accessory virulence determinants (Table 5). Although all bacteria were, by definition, negative for *bfpA*, nearly all were also negative for *efa1* and *lpfD*, the genes for factors that have been implicated as adhesins of some attaching-effacing strains of *E. coli* (19,25). Only two strains were positive for *astA*, which has also been suggested to contribute to the virulence of atypical EPEC (26).

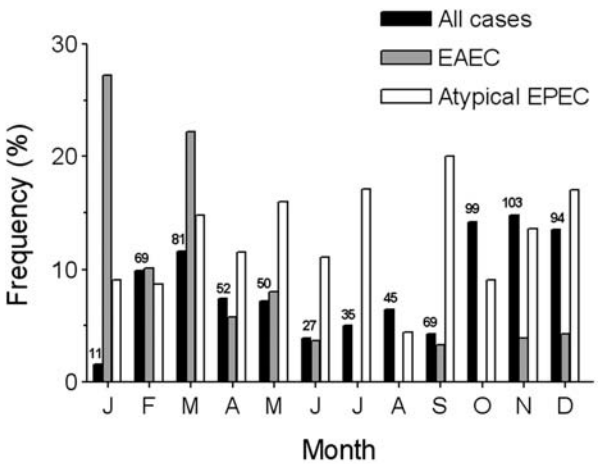

Figure 1. Seasonal incidence in gastroenteritis in the Melbourne Water Quality Study, 1998. Solid black bars indicate all cases of gastroenteritis as a percentage of the total, with the number of cases indicated above each bar. The frequencies of enteroaggregative *Escherichia coli* (EAEC) and enteropathogenic *E. coli* (EPEC) are expressed as a percentage of all cases examined each month.

Table 5. Characteristics of 22 isolates of atypical enteropathogenic *Escherichia coli* identified during this study

Strain	Source[a]	Serotype	Intimin type	Result of PCR[b]					Adhesion pattern[a]	FAS assay	HEp-2 cell invasion[c]
				bfpA	*aggR*	*efa1*	*astA*	*lpfD*			
W7	N	O161:H40	β	–	–	–	–	–	NA	–	0.01
W85-2	N	OR:H-	γ	–	–	–	–	–	AA	+	0.02
W114	N	O107:H8	ι	–	–	–	–	–	IA	+	0.02
W143	N	Ont:H5	ε	–	–	–	–	–	IA	+	< 0.01
W145	N	O55:H6	γ	–	–	+	+	–	AA	+	0.03
W154	N	O139:H14	β	–	–	–	–	–	AA	+	< 0.01
W185	N	Ont:H21	θ	–	–	–	–	–	IA	+	0.02
W208	N	Ont:H4	β	–	–	–	–	–	IA	+	0.08
W761	N	O124:H40	λ	–	–	–	–	–	NA	–	0.07
W902	N	O125:H6	α2	–	–	–	–	–	IA	+	0.06
W914	N	O125:H6	α	–	–	–	–	–	AA	+	0.07
W1040	G	O15:Hnt	β	–	–	+	–	+	LAL	+	0.10
W1056	G	O55:H7	γ	–	–	+	+	–	IA	+	0.77
W1068	G	O51:H49	α	–	–	–	–	–	AA	+	< 0.01
W1082	G	Ont:H6	β	–	–	–	–	–	AA	+	0.01
W1092	G	OR:H-	η/ε	–	–	–	–	+	IA	+	< 0.01
W1108	G	O172:H4	θ	–	–	–	–	–	IA	+	0.03
W1118	G	O126:H6	α	–	–	–	–	–	IA	+	0.01
W1120	G	Ont:H34	α	–	–	–	–	–	IA	+	0.01
W1134	G	Ont:H6	θ	–	–	–	–	–	NA	–	0.01
W1585	G	Ont:H40	θ	–	–	–	–	–	NA	–	0.02
W1706	G	Ont:H6	α	–	–	–	–	–	AA	+	0.01
E128012	C	O114:H2	β	–	–	+	–	+	IA	+	0.32

[a]N, no symptoms; G, gastroenteritis; C, control strain; Ont, O nontypable (O1–O181); Hnt, H nontypable (H1–H56); NA, nonadherent; AA, aggregative adherence pattern; IA, indeterminate adherence pattern; LAL, localized-like adherence pattern; FAS, fluorescent actin staining.
[b]PCR, polymerase chain reaction; +, positive; –, negative.
[c]Data are the number of bacteria recovered from HEp-2 cells after treatment with gentamicin as a percentage of the total number of cell-associated bacteria and are the mean of two separate assays.

Adhesion to HEp-2 Cells.

Typical EPEC adhere to HEp-2 cells in a distinctive pattern termed localized adherence, which requires the presence of Bfp (5). The 22 atypical EPEC strains isolated in this study showed variable patterns of adherence to HEp-2 cells, including aggregative adherence (7 strains) and a pattern previously termed localized-like adherence (1 strain) (27). Ten strains showed an indeterminate pattern of adherence, with small numbers of bacteria distributed apparently at random on the cell surface (Figure 2), and 4 strains were classified as nonadherent. The frequency of strains displaying each pattern of adherence was similar among isolates from patients with gastroenteritis and healthy persons. All atypical EPEC strains that adhered to HEp-2 cells, regardless of their pattern of adhesion, were positive in the FAS assay (Figure 3), which indicates that the LEE in these bacteria is functional. All nonadherent strains hybridized with DNA probes were derived from different regions of LEE, which suggests that they carry the entire pathogenicity island. We did not attempt to determine whether LEE was functional in these bacteria.

Previous reports of atypical EPEC have suggested that invasion of epithelial cells may contribute to the virulence of these bacteria (28). In this study, however, only one strain, W1056 (O55:H7), was able to invade HEp-2 cells to a noticeable extent (Table 5).

Discussion

The role of EPEC as a cause of diarrhea in children, particularly in developing countries, is now well established (2,3). The proven virulence determinants of EPEC include genes within the LEE, notably intimin (the outer membrane protein product of the *eae* gene), and Bfp, which is encoded by EAF (5). The key role of EAF in promoting the virulence of EPEC was established by Levine et al. (12), who showed that an EAF-negative derivative strain of EPEC, E2348/69, is markedly less virulent for adult volunteers than the wild-type strain. The same study showed that an atypical EPEC strain, E128012, which intrinsically lacks EAF, is also virulent in volunteers. This observation established that certain EPEC strains do not require EAF to cause disease. These intrinsically EAF-negative strains were originally called Class II EPEC (2,3) but are now more generally referred to as atypical EPEC. They are characterized by the presence of LEE and the absence of factors encoded by EAF, in particular, Bfp. In this way, atypical EPEC resemble EHEC, which are able to cause diarrhea despite their lack of Bfp. Indeed, persuasive

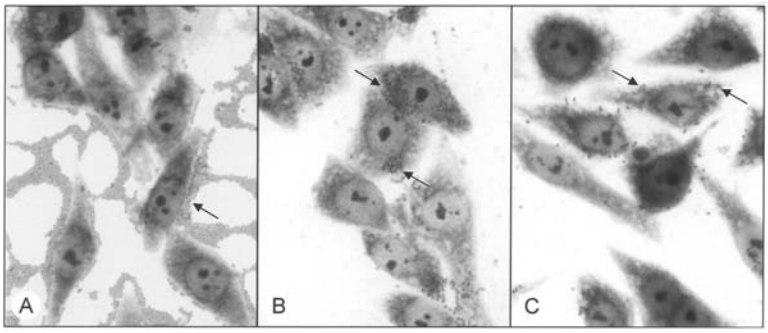

Figure 2. Patterns of adherence of atypical enteropathogenic *Escherichia coli* strains (arrows) to HEp-2 epithelial cells. A) aggregative adherence, B) localized-like adherence, and C) indeterminate adherence. Magnification x1,000.

evidence indicates that the most prevalent EHEC strain, serotype O157:H7, evolved from an atypical EPEC strain of serotype O55:H7 (29).

The aims of the WQS were to investigate the effect of household water treatment units on the incidence of gastroenteritis in Melbourne and to identify causative agents of gastroenteritis in the study population. The microbiologic investigations showed that the detection rate of EAEC (6.5%) and atypical EPEC (12.3%) in patients with diarrhea was greater than that of *Campylobacter* spp. (3%), *Salmonella* spp. (1.1%), adenovirus (1.1%), rotavirus (1.4%), *Cryptosporidium* spp. (1.6%), and *Giardia* spp. (2.5%) and was matched only by that of noroviruses (11.4%) (6,7,30).

Together, atypical EPEC and EAEC accounted for 19.3% of all cases; 21% of cases were attributable to all other bacterial, viral, and parasitic causes combined. However, the frequency of EAEC in patients with gastroenteritis and that in the baseline group without diarrhea was the same when matched for the time of year when the sample of feces was collected. In contrast, atypical EPEC was isolated significantly more often from patients with gastroenteritis than from those without symptoms, regardless of when the sample was collected. A subset of 22 randomly selected atypical EPEC strains was examined and found to be highly heterogeneous, which indicates that the high frequency of atypical EPEC in the study population was not the result of one or more outbreaks attributable to a small number of strains.

Despite the persuasive evidence of a volunteer study and reports of outbreaks of diarrhea with atypical EPEC (12,26,31), the role of atypical EPEC in disease is controversial. Originally, atypical EPEC were grouped with EPEC but were then segregated because they lack EAF. Justification for this division stemmed from the observation that EAF-bearing EPEC far outnumber atypical EPEC as the cause of infantile diarrhea in less-developed countries and of diarrhea outbreaks in general (3). In recent reports, however, from countries as diverse as Iran, Poland, South Africa, and the United Kingdom, atypical EPEC strains have outnumbered typical strains as a cause of gastroenteritis (32–35). Atypical EPEC were also more frequent than typical strains in aboriginal children hospitalized for diarrhea in the Northern Territory of Australia (36). These findings were reflected in the present study: 89 (94%) of *eae*-bearing strains identified in patients with gastroenteritis were atypical EPEC.

As for EPEC in general, atypical EPEC were originally incriminated as intestinal pathogens by virtue of their epidemiologic association with cases of diarrhea (2). Subsequently, these strains, which had been identified by serotype alone, were shown to be EPEC sensu stricto (37). Although atypical EPEC generally are serotypes which differ from EAF-positive EPEC (and other pathotypes of diarrheagenic *E. coli*), the 12 O-serogroups recognized by the World Health Organization as EPEC (i.e., serogroups

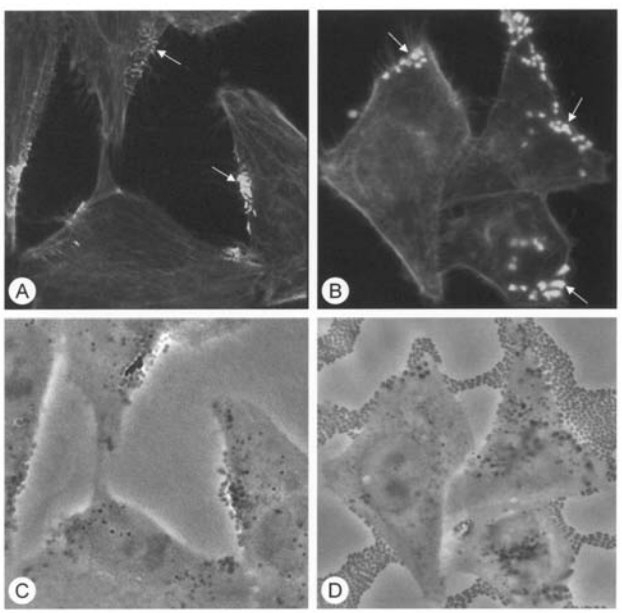

Figure 3. Fluorescent actin staining (FAS) assay for attaching-effacing capacity of atypical enteropathogenic *Escherichia coli* (EPEC) strains with different patterns of adherence to HEp-2 cells. Fluorescent micrographs of HEp-2 cells (A and B) incubated with strains of atypical EPEC showing localized-like and aggregative adherence, respectively, and then reacted with fluorescein-labelled phalloidin. Note the foci of intense fluorescence (arrows) associated with adherent bacteria, which were also visualized by phase contrast microscopy of the same microscope fields (C and D). Magnification x1,000.

O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158) include both typical and atypical varieties (3). Some of the atypical EPEC strains within these serogroups carry accessory virulence-associated determinants such as the EHEC hemolysin (commonly found in serotypes O26:H11 and O111ac:H8). Some strains also carry *astA*, the gene for enteroaggregative heat-stable enterotoxin, EAST1, which is frequently found in serotypes O55:H7, O119:H2, and O128:H2 (3). Atypical EPEC strains of non-EPEC serogroups generally do not express these factors. In the present study, none of the 89 atypical strains was positive for *ehxA*, which is required for the production of EHEC hemolysin, and although two strains (both serogroup O55) tested positive for *astA*, both carried the previously described mutations in this gene, which would preclude the synthesis of biologically active enterotoxin (38).

Unlike EAF-bearing strains of EPEC, which show localized adherence to HEp-2 cells, atypical EPEC show different patterns of adherence. Although some investigators have reported localized-like adherence as a predominant adherence pattern of atypical EPEC (28,39), only 1 of 22 strains investigated here displayed this phenotype. The low frequency of localized-like adherence in this study may reflect differences in serotype distribution, as localized-like adherence seems to be most prevalent in atypical EPEC strains in EPEC serogroups, such as O26, O111, and O119 (28), which were infrequently found in this study. Ten of the 22 strains examined exhibited an adherence pattern that was distinct from localized, aggregative, or diffuse adherence (3) and was termed indeterminate adherence (Figure 1). This pattern may have been termed diffuse adherence by other investigators, which would account for the relatively high frequency of diffusely adherent atypical EPEC in some reports and their absence from this study. Seven of 22 strains displayed aggregative adherence despite their lack of known sequences associated with the production of fimbriae of EAEC.

The virulence of atypical EPEC despite their lack of Bfp, which typical EPEC require to cause severe disease, suggests that these bacteria carry an adhesin analogous to Bfp that augments their ability to colonize the intestine. Previous studies, however, have shown only a low frequency of known *E. coli* adhesins, including aggregative adherence fimbriae, P fimbriae, S fimbriae, PAP pili, and afimbrial adhesins in atypical EPEC strains (40). We extended these findings to show that atypical EPEC are also mostly negative for Efa1 and long polar fimbriae (encoded by *lpfD*), which contribute to the adhesive capacities of some attaching-effacing strains of *E. coli* (19,25). Although atypical EPEC may carry an adhesin equivalent to Bfp, which remains to be discovered, these bacteria may use any known *E. coli* adhesins to bind to the intestine. This suggestion is supported by the marked heterogeneity of atypical EPEC in terms of adhesion pattern and the observation that adhesins other than Bfp can restore cell-binding capacity to EAF-cured strains of typical EPEC (41).

In conclusion, atypical EPEC were the most commonly identified pathogens in a study of community-acquired diarrhea in Melbourne. Infections with atypical EPEC occurred throughout the year and were significantly more common in children. Characterization of a sample of atypical EPEC isolates revealed that these bacteria were antigenically heterogeneous and generally did not belong to O-serogroups associated with EPEC. Further studies are needed to determine the frequency of these bacteria in other communities, their reservoir, mode of spread, and mechanisms of virulence.

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Syndromic Surveillance for Influenzalike Illness in an Ambulatory Care Network

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Conventional disease surveillance mechanisms that rely on passive reporting may be too slow and insensitive to rapidly detect a large-scale infectious disease outbreak; the reporting time from a patient's initial symptoms to specific disease diagnosis takes days to weeks. To meet this need, new surveillance methods are being developed. Referred to as nontraditional or syndromic surveillance, these new systems typically rely on prediagnostic data to rapidly detect infectious disease outbreaks, such as those caused by bioterrorism. Using data from a large health maintenance organization, we discuss the development, implementation, and evaluation of a time-series syndromic surveillance detection algorithm for influenzalike illness in Minnesota.

Rapid identification of a bioterrorism-related outbreak poses challenges to traditional public health disease surveillance (1). At the individual level, the nonspecific prodrome of many diseases caused by bioterrorism agents requires the disease to be recognized by an astute clinician (2,3). At the population level, an intentional release of a bioterrorism agent may require that disease clusters of syndromes, such as influenzalike illnesses (ILI), be recognized through recently developed nontraditional surveillance mechanisms, such as syndromic surveillance (4–7). For instance, increases in ILI shown by syndromic surveillance could indicate undiagnosed inhalation anthrax or pneumonic plague.

Various data sources can be used to construct syndromic surveillance systems. Existing patient data sources, such as emergency room chief complaints, ambulance dispatch data, and clinical diagnosis data, have been used (8–12). Metadata collection systems that incorporate emergency room syndromes, private practice billing codes grouped into syndromes, and veterinary syndromes also exist (7). Other existing data sources that are potentially suitable for syndromic surveillance include calls to poison control centers, over-the-counter and prescription medication sales (7,13), nurse help-line telephone logs (7,14), and absenteeism in schools (7). In this study, we evaluate the use of data from an ambulatory care clinic network to detect increases of ILI using a time-series autoregressive and cumulative sum (CUSUM)–based detection algorithm.

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Methods

Data Source

Data in this study are from the HealthPartners Medical Group (HPMG), which is a family of nonprofit Minnesota healthcare organizations that serves approximately 240,000 patients in the Minneapolis-St. Paul Metropolitan Area. HPMG is a current partner in the National Bioterrorism Syndromic Surveillance Demonstration Program (12).

Investments in technology infrastructure allow HPMG to digitally record International Classification of Diseases, Revision 9 (ICD-9) data from patient visits to network clinics within approximately 24 hours of a patient's initial visit. The need to develop validated and standardized methods for syndromic surveillance is a current challenge to the field. A framework for evaluating syndromic surveillance systems has been developed, which provides a general approach to comparing and contrasting aspects of syndromic surveillance systems (15). We have adapted some of these recommendations and have identified the following six criteria that should be satisfied before developing syndromic surveillance detection algorithms: 1) data are col-

lected and should exist for reasons other than bioterrorism surveillance; 2) data should be recorded and accessible in a recognized, consistent, and electronic format; 3) data should be available for analysis shortly after the patient's initial visit; 4) sufficient historical data sources should be available that represent a reasonably static and definable population; 5) syndromes should be validated against existing traditional data sources; and 6) thresholds set for these systems should achieve high sensitivity and positive predictive value. Based on the framework for evaluating syndromic surveillance systems, we assess the appropriateness of ambulatory-care encounter data from HPMG.

To satisfy the first criterion, these data are collected at HPMG shortly after the patient's initial visit; then identification is removed and the data transferred to the Minnesota Department of Health for analysis without additional work for physicians, clinic staff, or patients. For each scheduled, same-day, or urgent care patient encounter, ICD-9 codes are collected, recorded, and stored in a standardized electronic format, which fulfills the second criterion. Multiple ICD-9 codes may be recorded for each patient (e.g., 786.2 cough and 780.6 fever). The ICD-9 format consistency allows for reliable syndrome classification and analysis. Other possible sources of data that have been reliably classified into syndrome categories and could be used in this type of time-series analysis include chief complaint text fields and Health Level 7 (HL-7) messaging data (16). ICD-9 encounter data and nonidentifying demographic information are queried daily from the HPMG central patient database and sent to the Minnesota Department of Health through secure file transfer protocol. Fulfilling the third criterion, an advanced electronic patient-tracking system allows for >90% of the encounter data at HPMG to be available in HPMG databases within 24 hours of the clinic encounter. In addition to the daily transmitted data, historical encounter data beginning in April 1999 are used in the analysis. These historical data represent a consistently insured population within the clinic network with minimal immigration into or emigration from the HPMG network, thus satisfying the fourth criterion for this type of autoregressive time-series analysis. Had HPMG experienced substantial changes in its insured population, the underlying statistical assumptions necessary for this analysis would have been violated (17). Validation against a traditional data source, the fifth criterion, and the selection of an appropriate threshold, the sixth criterion, will be discussed below in the Validation section.

Models and Analysis

We used an autoregressive model (PROC AUTOREG) to model the square root of the daily counts of ILI to the HPMG clinics in a 3-year historical period (18). Sample SAS code with tests for autocorrelation and stepwise autoregression is provided in the online Appendix (http://www.cdc.gov/ncidod/EID/vol10/no10/03-0789_app.htm). The model closely resembles ordinary linear regression, but instead of the usual regression model, the following autoregressive error model is used:

$$y_t = x_t'\beta + \upsilon_t$$
$$\upsilon_t = -\varphi_1\upsilon_{t-1} - \varphi_2\upsilon_{t-2} - \ldots - \varphi_m\upsilon_{t-m} + \varepsilon_t$$
$$\varepsilon_t \sim IN(0,\sigma^2)$$

The notation $\varepsilon_t \sim IN(0,\sigma^2)$ indicates that each ε_t is normally and independently distributed with mean 0 and variance σ^2. By simultaneously estimating the regression coefficients β and the autoregressive error parameters ϕ_i, the model corrects the regression estimates for autocorrelation, a common problem in time-series data.

In the model, we include an indicator for weekend or weekday, an indicator of the day as a regular or national holiday, and indicators for a sine and cosine function for seasonal adjustment. The model also includes a seventh-order autoregressive error model, selected by stepwise regression. In each case, the terms contribute significantly to the fit of the model ($p < 0.05$).

The predicted residuals from this model are then analyzed by using the cumulative sums method (PROC CUSUM) (18,19). Initially used in the manufacturing industry, CUSUM has been used for *Salmonella* surveillance in the United States and for influenza surveillance in the United Kingdom (20–22). The method has properties making it well suited for disease outbreak detection. It can quickly detect small shifts from the process mean, provide estimates of when the change occurred, and estimate the magnitude of change (17,23–25).

Validation

Because syndromic surveillance attempts to identify disease outbreaks before a definitive diagnosis is made, assessing the validity of the ILI syndrome is difficult. The actual cause of many signals generated by this system may never be known because many patients are never requested to submit specimens for laboratory testing.

We assessed the validity of the HPMG ILI syndrome category by comparing ILI visits in the HPMG network to deaths from pneumonia and influenza in the core seven-county Minneapolis-St. Paul metropolitan area over the same time period. ICD-9 codes that describe ILI were selected (Table). We also associated increases in ILI with the known onset of influenza season by comparing influenza isolates and hospital laboratory data.

System Testing

Data pertaining to the incubation period for inhalational exposure to most potential bioterrorism agents are limit-

Table. Influenzalike illness ICD-9 codes selected for analysis[a]

ICD-9 code	Description
079.99	Viral infection
307.81	Headache, tension
372.30	Conjunctivitis
460	Rhinitis, acute
461.1	Sinusitis, acute, NOS
462	Pharyngitis
464.0	Laryngitis
464.4	Croup
465.9	URI
466.19	Bronchitis, acute
472.0	Rhinitis
477.9	Rhinitis, allergic
483.0	Pneumonia, mycoplasm
486	Pneumonia
496	COPD
487.1	Influenza
490	Bronchitis
493.00	Asthma/ROAD
493.90	Asthma
780.4	Dizziness
780.6	Fever
780.79	Fatigue/weakness/malaise
782.5	Cyanosis
784.0	Headache
785.6	Lymphandenopathy
786.07	Wheezing
786.09	Dyspnea
786.2	Cough
786.50	Pain, chest
787.02	Nausea
787.03	Vomiting

[a]ROAD, reversible obstructive airways disease; NOS, not otherwise specified; URI, upper respiratory tract infection; COPD, chronic obstructive pulmonary disease.

ed. Therefore, evaluation of this system used hypothetical scenarios in which additional ICD-9 counts were added to existing clinical data to determine the number of excess cases necessary to trigger a signal. In an approach adapted from Goldenberg, our hypothetical scenario uses data gathered from the only documented large-scale aerosol release of weapons-grade *Bacillus anthracis* spores (13).

In April and May 1979, an unusual outbreak of inhalational anthrax occurred in the city of Sverdlovsk in the former Union of Soviet Socialist Republics. The outbreak was originally ascribed to consuming contaminated meat, but investigation by Soviet and international scientists subsequently linked the outbreak to an accidental aerosolized release of anthrax spores from a nearby military facility (26). The inadvertent release, which may have contained as little as several milligrams of spores, caused 77 confirmed inhalational anthrax cases and 68 deaths in 43 days.

To test our model, we constructed three hypothetical scenarios on the basis of data available from the Sverdlovsk release. We made several assumptions in constructing these scenarios. First, a point-source release pattern similar to that observed in Sverdlovsk occurs in the downtown area of the Minneapolis-St. Paul metropolitan area during a weekday when most of the population is at work. This scenario will effectively disperse exposed persons to most clinics in the HPMG network, if one assumes that exposed persons will seek care at a clinic near their residence. Second, a subset of those exposed to the release will visit an HPMG clinic on the date of symptom onset and in a manner identical to those exposed in the Sverdlovsk release. This assumption effectively replicates the incubation periods experienced by those in the Sverdlovsk release and adds these additional cases to the daily totals of ILI observed in HPMG clinics. The third assumption increases the overall numbers of patients seeking care in the HPMG network from this exposure to 308 during a 43-day period, four times the number of confirmed ill in the Sverdlovsk release. The anthrax release and the resulting increase in ILI were modeled for three different time periods to determine the effect of season, day of the week, holidays, and naturally occurring ILI on the ability to detect the outbreak.

Results

Validation

The seasonal variation in HPMG ICD-9 ILI counts is similar to the variation in deaths from pneumonia and influenza in the core seven-county Minneapolis-St. Paul metropolitan area, as reported by the Minnesota Department of Health (Figure 1). To satisfy the fifth criterion for establishing syndromic surveillance, i.e., validating syndromes against existing traditional data sources, death data from April 10, 1999, to December 29, 2000, were compared to ILI ICD-9 counts over the same period. Visual comparison of these data in Figure 1 suggests that ICD-9 ILI counts rose several weeks before the peak in deaths. ILI syndrome validity was determined to be acceptable, as Pearson correlation results were significant between weekly influenza and pneumonia deaths and ILI clinical encounters in the same week (0.41) and the previous week (0.41).

Formal calculations of sensitivity and positive predictive value were not conducted in this study. Calculation of the appropriate threshold used in the detection algorithm was determined qualitatively by adjusting the model parameters to detect the onset of influenza season. Figure 2 illustrates a large and continuous signal that was retrospectively observed beginning on December 12, 2000, and continuing until December 25, 2000. This alarm corresponds to a large ILI outbreak in the Minneapolis-St. Paul metropolitan area and was possibly associated with increased influenza A and respiratory syncytial virus infection. A hospital in the HPMG network reported above

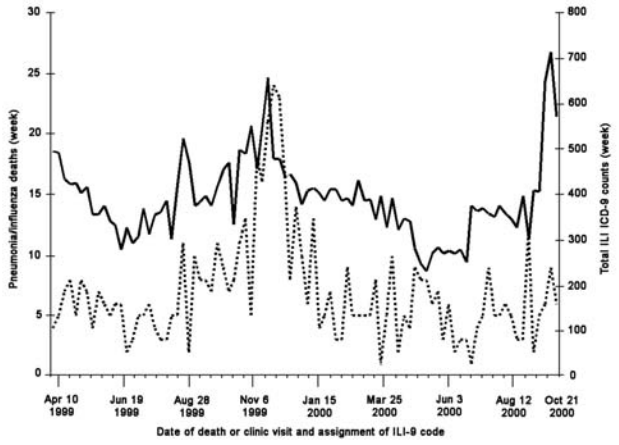

Figure 1. Weekly totals of HealthPartners Medical Group influenzalike illness ICD-9 counts (solid line) and Minneapolis-St. Paul metropolitan area weekly influenza and pneumonia deaths (broken line) April 10, 1999, through December 29, 2000.

average submission and testing of isolates corresponding to these organisms during December 2000 and January 2001.

System Testing

The hypothetical anthrax release was modeled at three different time periods beginning June 26, 2001, December 17, 2001, and April 1, 2002. The threshold for CUSUM in each scenario was calculated as 1.1812, which resulted in an average-run-length of 50. The outbreak that began in June was detected on June 30, 4 days after the release, with a CUSUM value of 3.09 and after 30 outbreak-associated ILI patients (11.9% increase above expected) visited the HPMG clinic network (Figure 3). The December outbreak was detected 7 days after the release with a CUSUM value of 2.33 and 130 outbreak-associated ILI patients (12.4% increase above expected) (Figure 4). The April outbreak was detected 5 days after the release with a CUSUM value of 2.00 and an additional 45 outbreak-associated patients with ILI (11.7% increase above expected) recorded in the clinic network (Figure 5).

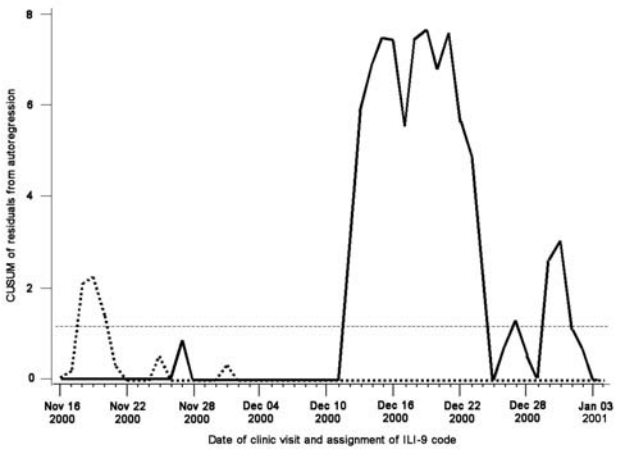

Figure 2. Cumulative sum (CUSUM) chart signaling a significant signal corresponding to a confirmed influenza A outbreak occurring December 2000 and January 2001. CUSUM decision interval (horizontal broken line); CUSUM chart signals 24 days earlier when the analysis is stratified by age: >65 years (dotted line) and all ages (solid line).

Discussion

Based on the six criteria we propose, we have attempted to construct a time-series syndromic surveillance system capable of detecting a bioterrorism or other public health event against the background of normal ILI clinic visits. Patient use patterns and seasonality have a considerable effect on the distribution of the dataset, an effect that must be considered when designing the autoregressive model.

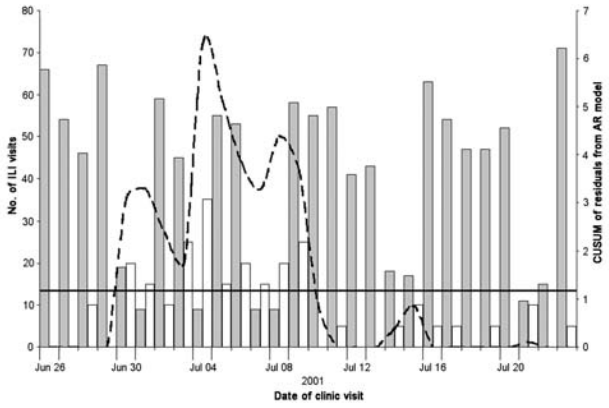

Figure 3. Cumulative sum (CUSUM) control chart of a hypothetical anthrax release occurring June 26, 2001. CUSUM of the residuals (broken line) is charted over the observed number of influenzalike (ILI) visits to the HealthPartners Medical Group (gray bars) and the additional outbreak-associated ILI cases (white bars). The system threshold, the CUSUM decision interval (solid line), is exceeded on June 30 and remains above threshold until July 9. With relatively low levels of ILI occurring in the summer months, this scenario demonstrates the ability of the system to detect increased ILI visits on weekdays and over the Fourth of July holiday.

Because the HPMG network offers same-day scheduling for its members, many patients do not seek care on the weekend, when only urgent care facilities are open. This delay results in an increased caseload on Monday, a situation that is further exacerbated on a 3-day weekend. The distribution of data is also affected by limited clinic access associated with holidays. The HPMG clinic network operates at a reduced capacity on New Year's Day, Memorial Day, Independence Day, Labor Day, Thanksgiving Day, Christmas Eve Day, and Christmas Day. These holidays often occur on different days of the week from year to year, and therefore generate lower-than-expected counts in the dataset. Additionally, ILI events occur with greater

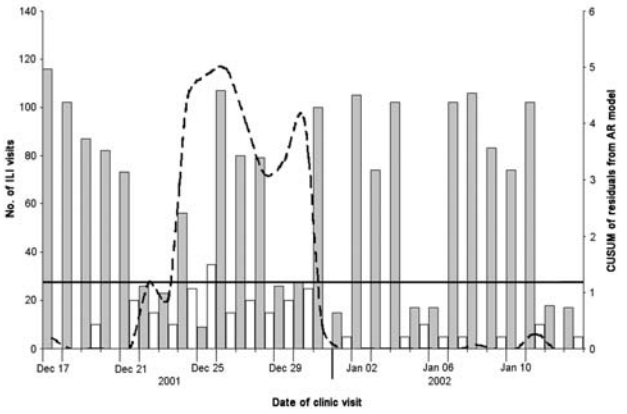

Figure 4. Cumulative sum (CUSUM) control chart of a hypothetical anthrax release occurring December 17, 2001. CUSUM of the residuals (broken line) is charted over the observed number of influenzalike (ILI) visits to the HealthPartners Medical Group (gray bars) and the additional outbreak-associated ILI cases (white bars). The system threshold, the CUSUM decision interval (solid line), is exceeded on December 24 and remains above threshold until December 30. With high levels of ILI occurring in the winter months, this scenario demonstrates the ability of the system to detect increased ILI visits during influenza season and over the winter holidays.

frequency in the winter, which generates a seasonal effect associated with the HPMG ICD-9 data.

Figure 1 shows general agreement between the distribution of ILI in the HPMG clinic network and influenza and pneumonia deaths in the greater metropolitan area during the same period. In the Minneapolis-St. Paul metropolitan area, a lag of 1 to 2 weeks occurs between time of initial signs and symptoms for ILI in HPMG clinics and an increase in influenza and pneumonia related deaths. This lag is less than that noted in other studies (27).

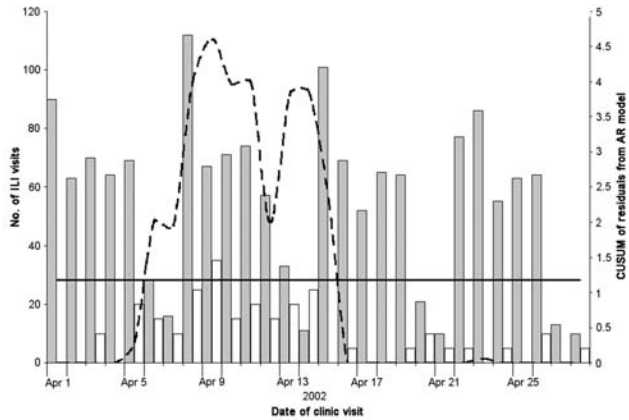

Figure 5. Cumulative sum (CUSUM) control chart of a hypothetical anthrax release occurring April 1, 2002. CUSUM of the residuals (broken line) is charted over the observed number of influenzalike (ILI) visits to the HealthPartners Medical Group (gray bars) and the additional outbreak-associated ILI cases (white bars). The system threshold, the CUSUM decision interval (solid line), is exceeded on April 6 and remains above threshold until April 15. In this scenario, an additional 45 cases of ILI over 5 days are necessary to push the CUSUM above threshold. This represents an 11.7% increase in ILI during that time period.

Influenza season in Minnesota is variable; onset ranges from early October through mid-January. Figure 2 illustrates a large, sustained increase of ILI beginning December 12, 2000. The Minnesota Department of Health Public Health Laboratory confirmed the season's first positive influenza isolate on December 13, 2000. This signal suggests that the rapid detection of ILI in the community is attainable by monitoring ICD-9 counts representative of ILI in a clinic network. When persons ≥65 years of age were separated into a distinct ILI syndrome category, a statistically significant signal is observed from November 18 to November 20. This increase in the ≥65-year category precedes the relatively large signal in the general population by approximately 3 weeks, demonstrating the utility of analyzing subsets of the patients as possible sentinel populations.

The ability of the system to detect additional bioterrorism-related cases is apparent in the hypothetical scenarios illustrated in Figures 3, 4, and 5. When background levels of ILI are relatively low, the system quickly detected additional cases associated with the anthrax release. At best, the system detected the outbreak only 2 days after the first case-patients began to visit the clinics. In winter months, when background ILI is higher, the system was slower to detect the outbreak-associated cases. In December 2001, a 5-day delay occurred between the appearance of symptomatic patients to the clinics and the recognition of the outbreak by the system. Twenty-five additional patients were seen at clinics on December 24, 2001, a holiday, and the system calculated a significant CUSUM alarm of 4.48. The ability of this system to detect the outbreak-associated cases at different times of the year, on weekends, and on holidays shows that the autoregressive model adequately controls for variance and autocorrelation in the dataset.

These scenarios demonstrate that the system possesses the ability to detect the cumulative sum of a small amount of additional counts. The practical success of this surveillance system is limited only by the availability and quality of the source data.

Conclusion

We have established criteria necessary for initiating syndromic surveillance for ILI and have demonstrated the effectiveness of our detection algorithm by using proxy data for a bioterrorism agent release and historical data for influenza. We believe that this approach to syndromic surveillance is useful in detecting increases in ILI.

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Disease Susceptibility to ST11 Complex Meningococci Bearing Serogroup C or W135 Polysaccharide Capsules, North America[1]

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Clusters of meningococcal disease caused by a hyperinvasive lineage of *Neisseria meningitidis*, the ST11 complex, bearing a serogroup C polysaccharide capsule, have been prominent in Europe and North America since the early 1990s. This situation has led to expensive public health measures for outbreak control and, finally, to the introduction of a serogroup C glyconjugate vaccine into the primary immunization schedule in the United Kingdom and elsewhere. ST11 complex meningococci may also express serogroup W135 polysaccharide capsules. We investigated the level of population immunity to this hyperinvasive clone in association with the appearance of outbreaks of meningococcal disease in southern British Columbia. We found that most adults and almost all children were apparently susceptible to infection with ST11 complex meningococci bearing both C and W135 polysaccharide capsules, which suggests that a vaccine program directed against only serogroup C meningococci may be insufficient to prevent hyperinvasive ST11 disease.

In 1919, George Heist and co-workers established that clotted blood from different persons varied in its ability to kill *Neisseria meningitidis* in a capillary tube. When Heist, whose blood had no bactericidal activity, acquired *N. meningitidis* infection, the link between serum bactericidal activity and resistance to meningococcal infection was proven (1). Nearly half a century later, Goldschneider et al. found that <20% of infants 1 year of age had anti–serogroup C meningococcal bactericidal activity in their blood, but >60% of teenagers and 75% of adults had protective titers (2). Disease rates were inversely related to the population bactericidal titers, with high rates of disease in young children and low rates in adults.

Groups of genetically related meningococci can be identified by using the electrophoretic mobility of cytoplasmic proteins (electrophoretic type; ET) or by nucleotide sequencing of "housekeeping" genes (sequence type; ST). During the 1990s, a clone of serogroup C *N. meningitidis* (ET-37 complex; ST11 complex) was responsible for outbreaks of meningococcal disease in the United States, Canada, and Europe, predominantly affecting teenagers and young adults and leading to repeated and massive public health interventions (3). In 1999, disease attributed to this clone led to serogroup C glyconjugate vaccine's introduction into the primary immunization schedule in the United Kingdom (4). From December 2000 to April 2001, a cluster of seven cases of invasive serogroup C meningococcal infection occurred in a community of 120,000 in southern British Columbia, Canada; five of the seven cases were in persons 18–27 years of age, which raises the possibility that more susceptible persons were found in this population than were previously inferred from data described by Goldschneider et al. 30 years ago (2).

During the 1990s, most ST11 complex *N. meningitidis* isolates in Canada bore an α2-9 N-acetyl neuraminic acid (serogroup C) capsule, but recent epidemics of meningococcal disease, particularly in Africa, have been associated with ST11 meningococci bearing the W135 capsule. This finding suggests that this hyperinvasive lineage might also spread to populations with low levels of population immunity against W135 capsule-bearing organisms. In addition, ST11 meningococci bearing serogroup B, and occasionally Y capsules, also occur (5). We examined population immunity to ST11 complex meningococci bearing serogroup C or W135 polysaccharide capsules.

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[1]These data were partially presented as a poster at the International Pathogenic Neisseria Conference in Oslo, Norway, 2002.

Methods

We obtained serum specimens from 175 healthy persons from southern British Columbia (Table) after the study protocol was reviewed by the University of British Columbia Clinical Research Ethics Board. Using these serum specimens, we examined the SBA, the ability of serum to kill meningococci when mixed with exogenous complement, against three target strains of *N. meningitidis*, AOBZ1379(c) (the outbreak clinical isolate from British Columbia; C:2a,P1.5; ET15), Z1582/FC978 (a Canadian clinical isolate from 2000 bearing the W135 capsule, W135:2a:P1.5,2) and C11 (60E; C:16:P1.7-1,1, a standard reference strain [2]), according to standard methods by using baby rabbit complement (Pel-Freeze Inc., Rodgerson, AR) as the exogenous source of complement (6). The highest serum concentration tested in the assay was 1:4, and a titer of 1:2 was assigned to sera with <50% killing at this concentration. The three bacterial strains were characterized by multilocus sequence typing, which confirmed the sequence-types as ST11, ST11, and ST345, respectively. We defined protection against serogroup C *N. meningitidis* as a serum bactericidal titer of ≥1:8 (4,7). Incidence data for rates of meningococcal disease were obtained from the British Columbia Center for Disease Control, Vancouver, Canada (Figure 1). The data are reported as geometric mean titers (GMTs) and as a percentage of the population higher than the protective threshold.

Results

The GMT of the bactericidal antibody against the outbreak strain of serogroup C, ST11 complex *N. meningitidis* was 1:2 in serum specimens from all children ≤18 years of age who were studied. In the group 19–29 years of age, the GMT rose to 1:4 and reached 1:7 in adults >30 years of age (Table). Three percent of children <18 years, and 19% of adults >19 years of age (median age 33 years), had serum bactericidal titers above or equal to the "protective" level (1:8) against this outbreak strain (Figure 1, Table). These data correlated closely with titers obtained by using strain C11, the standard reference strain used in serogroup C bactericidal assays (Figure 2). The reference serum CDC1992 (National Institutes for Biological Standards and Control) also produced identical SBA titers with both meningococcal strains.

The GMT of bactericidal antibody against the W135 strain of *N. meningitidis* rose steadily from 1:4 in those 18 months of age to 1:8, 1:16, 1:24, and 1:20 in those aged 4–6 years, 11–12 years, 16–18 years, and ≥19 years, respectively (Table). Sera tested from 20% of infants had bactericidal activity against the serogroup W135 ST11 complex organism, with titers ≥1:8 in up to 40% of serum samples from persons in the second decade of life and 44% in serum from adults. Rates of meningococcal disease in the population were inversely related to the population levels of bactericidal antibody for both serogroup C and serogroup W135 cases (Figure 1, Table).

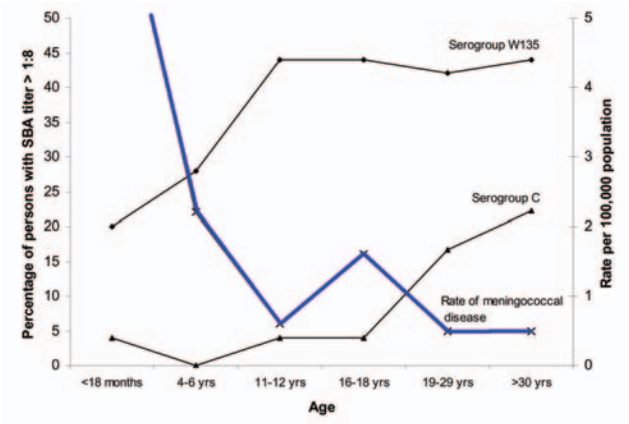

Figure 1. Incidence (cases/100,000/year) of meningococcal disease (average rates 1985–2000) in relation to serogroup C and W135 bactericidal antibody titers in British Columbia against a local ST11 outbreak isolate (AOBZ1379) and Z1582/FC978 (a Canadian clinical isolate from 2000 bearing the W135 capsule), respectively.

Conclusions

This study suggests that population immunity against hyperinvasive lineages of meningococci are low in a North American population. Protective serum bactericidal titers were present in 3% of children <18 years of age and 19% of adults (median age 33) against the outbreak strain of serogroup C, ST11 complex *N. meningitidis* (Figure 1). These bactericidal titers were low even though we used a complement source (baby rabbit serum) that is associated with higher SBA titers than the human complement used in Goldschneider et al.'s study in the 1960s, which found considerably higher levels of protection (2). Of note, 40% of meningococcal disease in the 1990s in Canada was caused by serogroup C meningococci (8). To exclude the

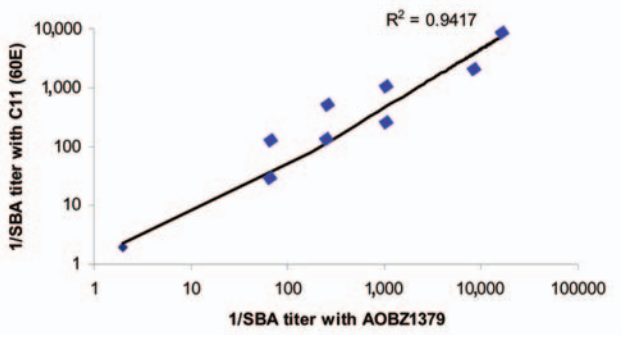

Figure 2. Correlation between bactericidal titers against an outbreak strain of serogroup C meningococcus (AOBZ1379) from southern British Columbia and the standard reference strain C11 from 27 serum specimens.

Table. Serum bactericidal activity against target strains of *Neisseria meningitidis*[a] for serum samples from different age groups[b]

	Serogroup C (ST11)				Serogroup W135 (ST11)			
Age	n	GMSBAT	95% CI	% ≥1:8	n	GMSBAT	95% CI	% ≥1:8
2 mo	24	2.4	2.3–2.7	4				
18 mo	25	2.1	2.1–2.3	4	25	4.3	3.8–5.0	20
4–6 y	25	2	2.0–2.0	0	25	8	6.6–9.7	28
11–12 y	25	2.4	2.2–2.5	4	25	16.5	13.4–20.1	44
16–18 y	25	2.3	2.2–2.4	4	25	24.3	19.4–30.3	44
19–29 y	24	4	3.5–4.6	17	7	21.5	8.1–57.2	43
>30 y	27	7	5.8–8.6	22	18	19.3	13.8–27.3	44

[a]AOBZ1379(c) (the outbreak clinical isolate from British Columbia) or Z1582/FC978 (a Canadian clinical isolate from 2000 bearing the W135 capsule).
[b]GMSBAT, geometric mean serum bactericidal assay titers; CI, confidence interval.

possibility that the outbreak strain was more resistant to serum bactericidal activity, we compared these data with those obtained by using C11, the serogroup C meningococcus used in 1969 by Goldschneider et al.; however, we found a close correlation (r = 0.97) between strains, which indicates no differences in serum resistance attributable to the different strains (Figure 2). Since these strains belong to different sequence types and they carry different subcapsular outer membrane proteins (C:16:P1.7-1,1 versus C:2a:P1.5), these data may indicate that antibodies against their common antigen (the serogroup C capsule) are more important than subcapsular antigens in the SBA. However, other subcapsular proteins common to both isolates may not have been identified, which could be responsible for this observation. By contrast, no correlation was seen (r = 0.2) between SBA titers achieved with 60E and Z1582/FC978, the ST11 W135 isolate, even though both isolates shared the same major outer membrane proteins (C:2a:P1.5); this finding further supports the importance of anticapsular functional antibodies in this assay.

Before routine immunization in the United Kingdom with serogroup C glyconjugate meningococcal vaccine was begun, Trotter et al. found bactericidal titers ≥1:8 in 10% to 20% of infants and 25% of adults (9). Similarly, Jones et al., found that 10% of university students appeared to have protective serum bactericidal levels (10). These U.K. findings are similar to our Canadian data that suggest that population immunity against serogroup C meningococcus may now be lower than previously described in countries without an immunization program.

We also found that 20% of infants had serum bactericidal activity against the serogroup W135 ST11 complex organism, with titers ≥1:8 in up to 40% in serum specimens from persons in the second decade of life and 44% in serum specimens from adults. The importance of anti-serogroup W135 serum bactericidal titers for protection is not well defined, but, extrapolating from serogroup C data, our results may indicate that 60%–80% of persons are susceptible to W135 disease. Less than 5% of laboratory-confirmed cases of invasive meningococcal disease in British Columbia are attributable to serogroup W135, but epidemic disease caused by ST11 complex organisms that bear the W135 capsule has been recognized in recent years and found to be associated with travel (11) and sub-Saharan African populations (12). Meningococci bearing the W135 polysaccharide capsule have been a relatively infrequent cause of sporadic cases of meningococcal disease in the 30 years since the first descriptions of this serogroup from cases in the U.S. army (13,14). However, in 2000 and 2001, an outbreak of disease occurred among pilgrims traveling to Mecca in Saudi Arabia for the annual hajj pilgrimage (14,15). This outbreak was caused by serogroup W135 meningococci from the ST11 (ET-37) complex (16), which was previously associated with hyperinvasive serogroup C disease. Since 2000, ST11 complex serogroup W135 meningococci have also appeared in sub-Saharan Africa and caused large epidemics (17). The association of epidemics of disease with meningococci of the hyperinvasive ST11 lineage that bears the W135 capsule is a cause for concern for populations with limited population immunity to these meningococci.

Figure 1 shows an apparent paradox: although the disease rate is decreasing substantially in persons <18 months to 11–12 years, little serologic evidence exists of a parallel rise in protection, i.e., the geometric mean SBA titers are almost constant, and the percentage of children with SBA titers <1:8 in all age groups up to those 16–18 years remains low. This finding could indicate that SBA lacks sensitivity (perhaps protection occurs before a titer of 1:8 is reached) or that protection rises through immune mechanisms other than the bactericidal combination of antibody and complement (such as osponophagocytosis). A decrease in exposure to serogroup C meningococci or a reduction in other cofactors that lead to invasive disease may also occur in this age group.

ST11 lineage meningococci may also bear serogroup B capsules, and evidence exists that a switch between serogroups may occur naturally in populations (18,19), perhaps a process that is favored by population immunity. Whether immunization with vaccines that target serogroup C capsule–bearing ST11 complex meningococci will favor the evolution of B or W135 ST11 complex bacteria in the

next few years is not clear. No evidence of the emergence of serogroup B ST11 complex meningococci has been reported in the United Kingdom since the serogroup C glyconjugate vaccine was introduced in 1999. However, in Spain, ST11 serogroup B meningococci have emerged since vaccine introduction, which raises the possibility that, in some circumstances, immunologic pressure on meningococcal populations by vaccine may lead to capsule switching (20).

Recent data after the serogroup C glyconjugate vaccine was implemented in the United Kingdom strongly suggest that SBA titers ≥1:8 are required for protection against serogroup C meningococcal disease (4,7). Data presented here indicate that a high proportion of adults, and almost all children, may be susceptible to disease caused by the hyperinvasive ST11 clone of serogroup C lineage that is prevalent in North America. These findings support the value of the childhood immunization program with serogroup C meningococcal glyconjugate vaccines, implemented in British Columbia, Canada, since September 2003. However, the propensity of this clone to express capsular polysaccharides other than serogroup C, including W135, and the apparently low population immunity for bacteria bearing the W135 capsule suggest that monovalent serogroup C vaccines may be insufficient to control this widely distributed hyperinvasive lineage of meningococcus, the ST11 complex.

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Genetic and Transmission Analysis of *Helicobacter pylori* Strains within a Family[1]

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To look for evidence of intrafamilial infection, we isolated 107 *Helicobacter pylori* clones from biopsied specimens taken from both parents and four children. We compared the sequences of two housekeeping genes (*hspA* and *glmM*) from these clones with those of 131 unrelated strains from patients living in different geographic regions. Strain relationships within the family were determined by analyzing allelic variation at both loci and building phylogenetic trees and by using multilocus sequence typing. Both *hspA*- and *glmM*-based phylogenetic trees showed East Asian and African branches. All samples from family members showed natural mixed infection. Identical alleles found in some strains isolated from the children and parents, but not in the strains isolated from unrelated patients, demonstrated that strains have circulated within the family. Several mechanisms, such as point mutations, intragenic recombination, and introduction of foreign (African) alleles, were shown to enhance strain diversity within the family.

Helicobacter pylori is the major cause of chronic gastritis and peptic ulcers and must be treated to prevent relapse (1). *H. pylori* infection is considered a risk factor for developing gastric carcinoma (2,3). *H. pylori* strains appear to be spread by person-to-person contact (4), and DNA fingerprinting has provided evidence of transmission between family members (5–9). Clonal descent has been demonstrated by comparing alleles of genes such as *vacA*, *flaA*, and *flaB* of isolates infecting members of the same family (10–12) and by sequencing three housekeeping genes (*ureI*, *atpA*, and *ahpC*) (13). However, in all these studies, only one strain from each biopsied specimen was studied.

H. pylori is one of the most genetically diverse bacterial species, displaying from 2.7% to 8.0% of DNA sequence polymorphism (14–16). This diversity originates from both the clonal nature of the species and interstrain recombination events (17–19). Analysis of the sequences of housekeeping genes (*atpD*, *scoB*, *glnA* and *recA*) showed that strains cluster according to their geographic origins (15,20). Strains from the United States, Latin America, and Europe differ from those that are predominant in East Asia, coastal China, Hong Kong, Japan, south Asia (India), and Africa (20–22).

We estimated the allelic diversity of 20 isolates taken from two locations (fundus and antrum) in the stomach of each member of a family and studied person-to-person transmission within this family. To assess the genetic diversity and relationships between the isolates, we sequenced two housekeeping genes (*glmM* and *hspA*). These two genes are present in all *H. pylori* isolates and have been shown to be good tools to distinguish between isolates (23,24); g*lmM* sequences appear to be relatively well conserved between strains (23), whereas *hspA* sequences show enough variability to test geographic clustering (24)

Materials and Methods

Participants, Gastric Biopsy Samples, and Related *H. pylori* Isolates

The family consisted of two parents and four children; child 1, child 2, child 3, and child 4 were 14, 12, 8, and 2 years of age, respectively. The parents were from Algeria, and all children were born in France. The two parents had gastritis and the children had abdominal pain. Child 1

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[2]These authors share senior authorship.

(index case-patient) was given appropriate triple therapy (lansoprazole plus amoxicillin plus clarithromycin for 7 days), according to the results of antimicrobial drug susceptibility testing. Despite treatment, this child remained infected with *H. pyloi*, which suggested treatment failure or reinfection. Informed consent was obtained from each adult and from the two eldest children. Parental consent was obtained for each child.

Biopsy samples were taken from the corpus and antrum of the stomach during endoscopic testing. Culture was performed as previously described (23). The Etest analysis (AB Biodisk, Solna, Sweden) showed that all isolates were susceptible to clarithromycin. When possible, 10 independent colonies were randomly selected from each primary culture and subcultured. A total of 107 independently subcultured isolates were stored as frozen suspensions. Repetitive sequence analysis previously found that freezing or subculturing strains had no effect on the stability of the *hspA* and *glmM* sequences.

Unrelated *H. pylori* Isolates

Epidemiologically unrelated *H. pylori* isolates were collected from persons who underwent gastroduodenal endoscopy in various gastroenterology departments. Nineteen of the patients originally from Hong Kong (Queen Mary Hospital), 9 were from Senegal (C.H.U. Le Dantec, Dakar), 25 were from Venezuela (Facultad de Medicina, Universidad de los Andes, Merida), 22 were from Sweden (Karolinska Hospital, Stockholm), 18 from Iran (Pasteur Institute of Iran, Tehran), and 32 were from France (Saint Vincent de Paul Hospital, Paris). Strains Ovx 34, Takada 112/3 (isolated from a monkey), X47-2AL (isolated from a cat), 26695 (ATCC700392), J99 (ATCC700824) (16), 85P, and N6 (23) were used as reference strains.

Molecular Techniques

Chromosomal DNA was extracted from 48-hour-old confluent cells by using the QIAamp Tissue Kit (Qiagen, Chatsworth, CA), according to the manufacturer's recommendations. A 487-bp segment containing the 384-bp *hspA* gene and a 294-bp fragment of the *glmM* gene was amplified by polymerase chain reaction (PCR) (24,25). Each purified PCR product was fully sequenced on both strands with an ABI310 automated DNA sequencer (Perkin-Elmer).

Computer Analyses

Multiple DNA sequences were aligned with the CLUSTAL V program (25). Phylogenetic analyses were performed with the PAUP* software package, version 4.0 (26). Sequence distance matrices were established in pairwise comparisons by using the Kimura algorithm and a transition/transversion ratio of 3.88 and 4 for the *hspA* and *glmM* genes, respectively. Phylogenetic trees were constructed by the neighbor-joining method (27). Maximum-parsimony trees were obtained by 1,000 random addition heuristic search replicates without the branch-swapping option. A maximum-likelihood analysis was performed with the HKY85 model, which calculated the transition/transversion ratio and estimated the shape parameter of a γ distribution of rate variation among sites (28). Significance was evaluated by the jacknife method. Split decomposition was carried out with the Splits Tree program, version 2.4, (http://bibiserv.techfak.uni-bielefeld.de/splits/) with pairwise distance estimated with the Kimura model, to detect phylogenetic incongruence and show how recombination might affect the evolutionary relationships between *H. pylori* strains. Multilocus sequence typing (MLST) tools were used to delineate clusters of strains by using sequence output available from the MLST Web site (http://mlst.zoo.ox.ac.uk). Clusters of strains or clonal complexes were defined with the BURST algorithm on the MLST Web site. The sequences obtained during this study were assigned the following EMBL accession numbers: for *glmM* AJ809447–AJ809497 and for *hspA* AJ809893–AJ810031.

Results

We isolated and subcultured 107 *H. pylori* colonies from the antral and corpus biopsy samples collected from the family members (9 or 10 individual colonies per sample, except for child 2, for whom the antral biopsy culture was negative). We sequenced the *hspA* and *glmM* genes of all 107 colonies. The multiple alignments showed 11 different alleles for *hspA* (designated H1a,b,c,d; H2a,b,c,d; H3a,b,c) and six different alleles for *glmM* (designated G1; G2a,b; G3a,b; G4). Each strain was named by the combination of the *hspA* and the *glmM* alleles (e.g., H1d-G1 for an isolate harboring the H1d *hspA* allele and the G1 *glmM* allele, Figure 1). All family members had a natural mixed infection.

We sequenced a 357-bp DNA region containing the entire *hspA* gene of 131 epidemiologically unrelated strains isolated from patients from different countries. The 19 Hong Kong strains contained a specific 9-bp signature, coding for [Thr-Asp-Ser] or [Thr-Asn-Ser] at positions 289–297 (*H. pylori* 85P, numbering system) of *hspA* (Figure 2). Sixteen of the Hong Kong strains belonged to the same branch of the *hspA*-based neighbor-joining tree (5 of these 16 Hong Kong strains are represented in Figure 3). Eight of the nine strains isolated in Dakar were grouped on the same branch as four strains from patients from Tunisia, Morocco, Algeria, and Senegal. This "African branch" was also visible on the parsimony consensus tree (data not shown). The African branch strains carried a specific

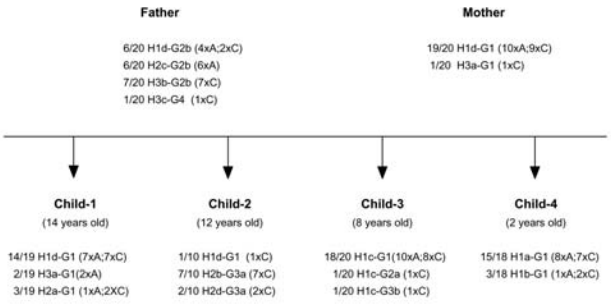

Figure 1. Number and genotypes of isolates from members of the family. The *hspA* and *glmM* alleles are designated by H and G, respectively. The alleles are numbered according to the cluster to which they belong on the phylogenetic trees (Figure 4). Small letters were assigned to the alleles that belong to the same cluster and differ by point mutations. (n x A; m x C), which shows the number (n or m) of colonies originating from the antrum (A) and the corpus (C), respectively.

15-bp signature, coding for [Asp/Glu-His-Lys-His-Ala], at positions 310–324 of *hspA* (Figure 2). The other branches of the *hspA* phylogenetic trees consisted of isolates from patients of different ethnic origins.

We sequenced the *glmM* genes of 47 randomly selected isolates. The *glmM* phylogenetic trees based on the neighbor-joining (data not shown) and maximum likelihood methods (Figure 4) grouped most of the sequences from the Hong Kong strains in the same branch. Similarly, the *glmM* gene sequences of the strains from Senegal, Morocco, and Algeria formed an African branch, as did the *hspA* sequences.

Phylogenetic analyses of the *hspA* sequences of all the isolates (family plus different geographic regions) indicated three clusters (H1, H2, H3) (Figure 4). The H1a, H1b, H1c, and H1d allelic sequences belonged to the H1 cluster, which was present in all isolates from members of the family; these four alleles had nonsynonymous mutations at both ends of the gene. Cluster H2 included the H2a–H2d alleles, found in isolates from child 1, child 2, and the father; these alleles differed by six synonymous mutations (positions 20, 77, 89, 98, 131, and 188; 85P numbering system) and a nonsynonymous mutation at the 5′ end. Both the H1 and H2 clusters belonged to the African branch. Cluster H3 contained the three alleles H3a, H3b, and H3c, found in isolates from child 1, the father, and the mother; these alleles differed by a nonsynonymous mutation (5′ end) and a synonymous mutation (position 164). This third cluster was included in a heterogeneous group of strains isolated from humans of diverse ethnic origins.

Four clusters of *glmM* sequences (G1 to G4) were observed in the global phylogenetic tree (Figure 4). Allele G1 was strictly identical in strains isolated from the four children and the mother. The G2 cluster included the G2a sequence found in isolates from child 3 and the G2b sequence found in those from the father, differing by one synonymous mutation (position 21). Cluster G3, grouping the G3a allele from strains from child 2 and the G3b allele from those of child 3 that differed by one synonymous mutation (position 129), was included in the African branch. Finally, allele G4, represented by a single sequence from a father-derived strain, showed no close relationships with any of the other DNA sequences included in this study.

Several alleles of strains isolated from the parents and children belonged to the same clusters on the phylogenetic trees (Figure 4), and alleles within each cluster showed only isolated mutations. Comparison of the 3′ end sequences of the *hspA* gene showed a clear mosaic structure (Figure 2). A low shape parameter (0.42) of the γ distribution, which characterizes the heterogeneity of rate variation among sites of *hspA* sequences, was obtained. All of these facts led us to conclude that intragenic recombination events enhanced allelic diversity.

The diversity of allele associations allowed us to demonstrate that lateral gene transfer took place between strains within the family. For example, the H1c allele was

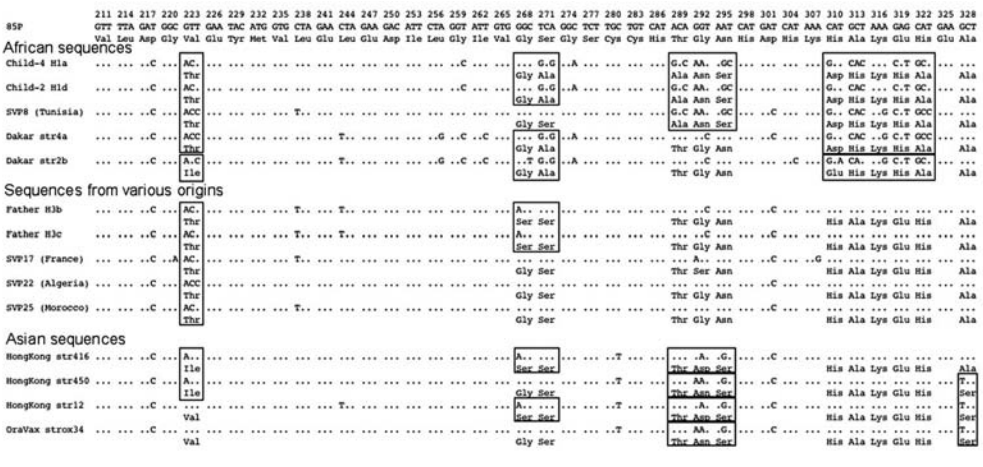

Figure 2. Mosaicism of the *hspA* 3'-end sequences.

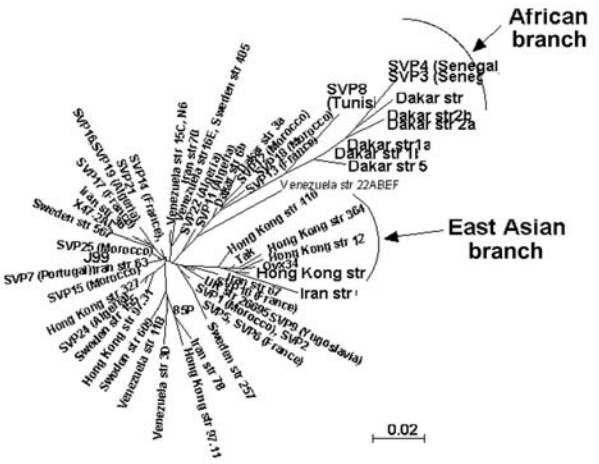

Figure 3. Neighbor-joining unrooted dendrogram for *hspA* sequences. The tree includes only 61 of the 131 *hspA* sequences representative of each cluster previously obtained from the global phylogenetic analysis. The scale bar indicates the number of substitutions per site according to the HKY index. Sequence names correspond to the geographic region of isolation followed by the strain number. SVP, Saint Vincent de Paul Hospital, Paris, France. The ethnic origin of French patients is mentioned in brackets when known.

associated with three different alleles (G1, G2a, and G3b) in the 20 strains from child 3 (Figure 1). In the same way, the G1 allele found in strains from the mother was associated with six *hspA* alleles (H1a, H1b, H1c, H1d, H2a, and H3a) in the strains isolated from the children. In these two examples, *hspA*- and *glmM*-associated alleles belonged to three genetically distant clusters. Thus, the two genes were acquired at different times even though they are located on the same chromosome (Figure 4). In addition, examination of phylogenetic trees of epidemiologically unrelated strains showed that some of the family alleles originated in Africa (H1a–H1d, H2a–H2d, G3a, and G3b) (Figures 3–5). The G3a and G3b alleles belonging to the African branch were only found in strains isolated from child 2 and child 3.

Analysis of strains from family members indicated four clonal complexes (A, B, C, D) (Figure 5). Strains harboring allele G1, found in six different combinations (H1aG1, H1bG1, H1cG1, H1dG1, H2aG1, H3aG1) or H1c, found in three different combinations (H1cG1, H1cG2a, H1cG3b), formed the main clonal subgroup, which included strains circulating between the mother and the children. The strains isolated from the father belonged to two subgroups (B and D). Subgroup D consisted of the strain H3cG4, containing a *glmM* allele of unknown origin, whereas subgroup B contained only the father-derived strains that harbored the G2b allele. The last subgroup (C) contained two strains isolated from child 2, characterized by two *hspA* and *glmM* African alleles (H2a, H2d, G3a).

Discussion

Almost every *H. pylori* strain from the family and from patients living in different geographic regions had its own, unique DNA sequence (15,22). The level of synonymous divergence between the *hspA* and *glmM* alleles was similar to that observed for *vacA*, *flaA*, and *flaB*, whereas the level of nonsynonymous divergence for these two genes was slightly higher than the highest rate previously observed (2.9% versus 0.3%–2.5%) (12). This observation suggests that the two chosen DNA fragments (*hspA* and *glmM*) correspond to domains with a high level of mutations and encode proteins with few functional constraints.

By examining 10 colonies from each gastric biopsy sample from the antrum and fundus of each of the family members, we identified 11 different *hspA* alleles and six different *glmM* alleles (Figure 1). Phylogenetic analysis allowed us to map these different *hspA* and *glmM* alleles in three and four distinct branches of their respective trees, which suggested that they originated from different strains (Figure 4). The strain diversity seemed higher in the corpus than in the antrum (Figure 1). With the exception of the father, who was equally colonized (30%) by three of the four isolates, all family members appeared to be infected by a dominant strain (>70%). This multicolonization in a North African family is in agreement with data reporting that multicolonization is more frequent in countries in which *H. pylori* infection is highly prevalent (29,30).

The phylogenetic analyses based on the *hspA* and *glmM* sequences of strains from several regions of the world allowed us to build neighbor-joining and maximum

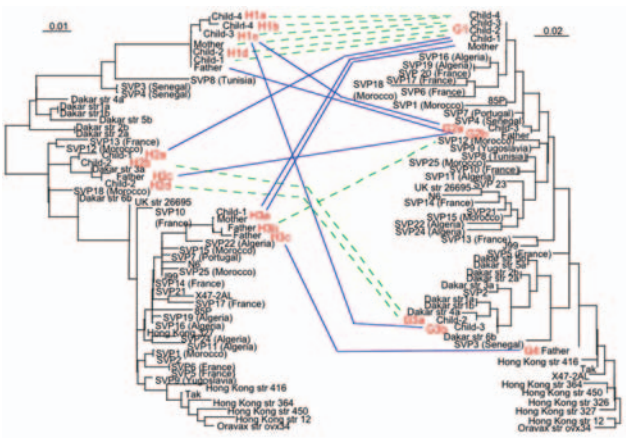

Figure 4. Relationships and genetic transfer hypotheses for the *hspA* and *glmM* alleles from *Helicobacter pylori* strains infecting members of the family. The phylogenetic trees based on *hspA* (left) and *glmM* (right) sequences were built by the maximum-likelihood method. The *hspA* sequences shown here originate from the same 47 strains from which the *glmM* sequence was determined. The names given to the sequences for family genes correspond to the name of the family member followed by the allele number. Dashed lines suggest coevolution of genes. Solid lines suggest genetic transfer.

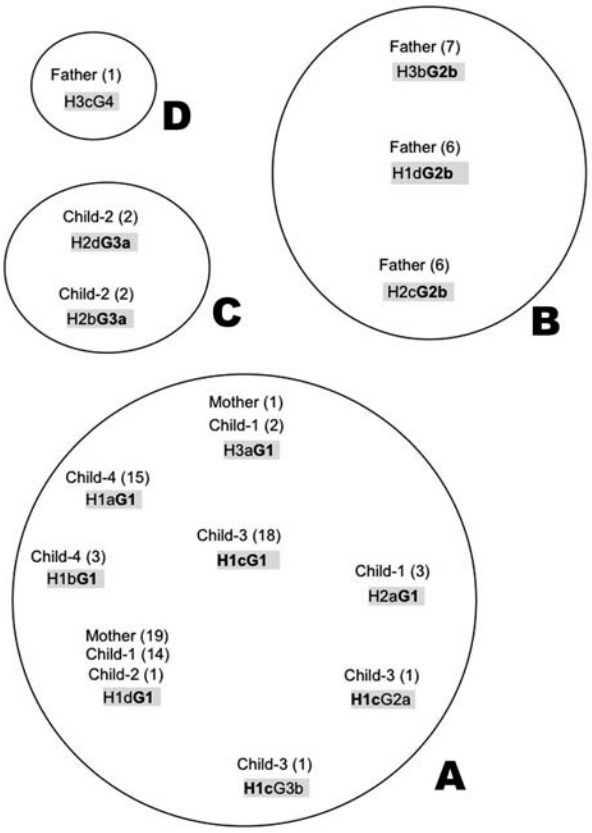

Figure 5. Multilocus sequence typing (MLST) analysis of family isolates. Clonal complexes were defined according to the alleles found associated with the highest number of different combinations among family strains. The names of clones are indicated by a letter: A, B, C or D. Alleles in bold are shared.

likelihood trees. Several nodes were not defined on the parsimony consensus trees, which indicates many polytomies. This finding suggests a high level of homoplasy, resulting from a high level of parallel and convergent mutations that could be the result of recombination events (15). However, some of the clusters in these phylogenetic trees were reproduced with distance and maximum likelihood building algorithms and with the jacknife sampling method, which indicates the robustness of some of the nodes. Reproducible deep-branching clusters were associated with the geographic specificity of strains (Hong Kong or Africa). An East Asian branch has previously been reported for *hspA*, *glmM*, *vacA*, and other housekeeping genes (15,22,31) and for the *cag* pathogenicity island of *H. pylori* (32). We confirmed the clustering of East Asian strains and provided evidence in support of the recently proposed clustering of African strains (22).

Within the family, *hspA* and *glmM* alleles belonged to different clusters. Each of these individual groups contained two to four alleles that differed by a few mutations, consistent with genetic drift as previously described in *H. pylori* (33). Furthermore, genetic recombination events occurred between strains colonizing the family members (Figure 4). This study demonstrated that intragenic recombination events occurred during the evolution of the *hspA* and *glmM* genes, which resulted in mosaicism, network-like structures, or both (Figure 2), as has previously been reported for *vacA*, *flaA*, and some housekeeping genes (12,34,35). The discrepancies in the phylogenetic positions of a given strain based on the sequences of the *hspA* and *glmM* genes illustrate that lateral gene transfer has occurred among strains circulating within the family.

Some of the children were infected with strains harboring African alleles. We can speculate that foreign strains were introduced into the family through the children and added heterologous DNA that further increased the genetic diversity. However, we cannot exclude the possibility that, despite the large number of isolates from each family member, we undersampled the population and missed some alleles present in other isolates.

This study showed that all family members had mixed infections. Some of the alleles in the family strains were phylogenetically distinguishable, which allowed us to demonstrate conclusively the circulation of strains and the genetic exchange between family strains and confirming the intrafamilial dissemination of *H. pylori*. DNA fingerprinting methods have previously described heterogeneous populations of *H. pylori* clones derived from the culture of biopsies taken from a single patient (13,15). Randomly amplified polymorphic DNA analysis, PCR-restriction fragment length polymorphism, ribotyping, and pulsed-field gel electrophoresis have also demonstrated the clonal dissemination of strains within a family, concluding that family cross infection occurred or that the family members were infected by a common source (5,8,13,20). However, none of these studies involved sequencing genes from 20 individual colonies, which impeded the detection of minor variations between strains.

By sequencing the *hspA* and *glmM* genes of 20 isolates for each person from a family, we highlighted the involvement of some of the evolutionary mechanisms previously described in epidemiologically unrelated strains. This finding may clarify the mode of transmission and the evolution of the *H. pylori* genome.

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Dr. Raymond is a microbiologist and responsible for the bacteriology laboratory of a pediatric hospital. Her research interests include the study of *H. pylori* infection in children, particularly in the diagnosis and epidemiology of infection. She works in collaboration with researchers at the Pasteur Institut of Paris.

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Geographic and Temporal Trends in Influenzalike Illness, Japan, 1992–1999

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From 1992 to 1999, we analyzed >2.5 million cases of influenzalike illness (ILI). Nationwide influenza epidemics generally lasted 3–4 months in winter. Kriging analysis, which illustrates geographic movement, showed that the starting areas of peak ILI activity were mostly found in western Japan. Two spreading patterns, monotonous and multitonous, were observed. Monotonous patterns in two seasons featured peak ILI activity that covered all of Japan within 3 to 5 weeks in larger epidemics with new antigenic variants of A/H3N2. Multitonous patterns, observed in the other five seasons, featured peak ILI activity within 12 to 15 weeks in small epidemics without new variants. Applying the kriging method allowed better visualization and understanding of spatiotemporal trends in seasonal ILI activity. This method will likely be an important tool for future influenza surveillance in Japan.

Influenza is a highly contagious acute respiratory disease that has caused global epidemics and pandemics. Pandemics in the 20th century have occurred at intervals of 11 to 39 years (1–3). The World Health Organization has requested each member state to produce a pandemic plan. The phasing and geographic spread of influenza pandemics have important implications for future planning, and complete global spread is now likely to occur in ≤6 months, as a result of increased travel and urbanization (4).

The National Epidemiological Surveillance of Infectious Diseases in Japan features sentinel surveillance for 27 infectious diseases, including influenza (5,6). To better understand the movement and velocity of influenza epidemic spread from 1992 to 1999 in Japan, we used a geographic information system (GIS) with generated weekly surveillance data. We focused on the kriging method to illustrate and clarify spatiotemporal relationships in epidemiologic research, e.g., for rotavirus and influenzalike illness (7–9).

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Methods

Influenza Surveillance System in Japan

The systematic surveillance of influenza and influenzalike illness (ILI) as notifiable diseases under Infectious Disease Control Law began in 1981 in Japan. Each ILI case is defined on the basis of a sudden fever ≥38°C, respiratory symptoms, and myalgia. The number of patients with ILI is reported on a weekly basis from ≈2,400 sentinel pediatric and general physicians and 663 health centers throughout Japan. The number of sentinels is decided on the basis of the size of the population of the health center area where they serve: a health center with population <75,000 would have one sentinel, a population 75,000–125,000 would have two, and populations >125,000 would have three + [(population − 125,000)/100,000] sentinels (10). Recruitment is on a volunteer basis. Sentinels forward clinical data to ≈60 prefectural or municipal public health institutes, and data generated are electronically reported to the Infectious Disease Surveillance Center in the National Institute of Infectious Diseases (Tokyo) (5,6).

Geographic Analysis

We analyzed surveillance data from 1992 to 1999 for 46 prefectures, excluding Okinawa Prefecture, which is approximately 800 km from the four major islands of Japan. To combine data from all prefectures and examine trends at the national level, we calculated the number of reported ILI cases per sentinel per week after adjusting the epidemic curves with a 5-week unweighted moving aver-

age (reported ILI cases per sentinel per week [RC/S/W]) as an indicator of ILI activity. This procedure smoothed the data and simplified identification of the seasonal peak (7). The peak week during each influenza season was defined when the greatest unweighted moving average was observed in individual prefectures. For the time scale of geographic analysis, the first week was defined when the first peak was observed in any of the prefectures during the season; subsequent weeks were then numbered accordingly.

For the spatiotemporal spread of the 1992–1999 epidemics in Japan, we used the kriging geostatistical method to estimate point values by using surrounding, known point values (11,12). The address of the prefecture government was used as the representative site of prefecture surveillance data, and unweighted moving average for each prefectural peak week was applied after adjusting the time scale. Kriging uses a weighted moving average interpolation to produce the optimal spatial-linear prediction. The estimated kriging weight matrix is a product of the inverse covariance weight matrix and the distance matrix (11,12).

To make kriging maps as contour maps showing the timing of peak ILI activity, we performed the following steps. First, we created an empiric semivariogram to examine the structure of data. The empiric semivariance is 0.5 times the difference squared, when Euclidean distance is used. Second, this semivariogram estimated the theoretical model parameters through a weighted least-squares technique. The data showed a spherical pattern. Next, the weights were determined by incorporating the spherical pattern of covariance. Finally, we estimated the values at unmeasured points and made filled-contour maps from the kriging weights for the measured values. The isobars on the contour maps represent interpolated time of peak activity distributed spatially and were placed at 1-week intervals. All procedures were carried out on ArcGIS 8.2 (ESRI, Redlands, CA) and Geostatistical Analyst (ESRI) for Windows.

Statistical Analysis

To ascertain the relationship between epidemic scale and velocity of spread, we used three parameters. greatest number of ILI cases, increasing-to-peak period, and nationwide peak-duration. The first parameter was the greatest number of ILI cases, defined as the greatest number of RC/S/W in each prefecture The second parameter was the increasing-to-peak period, defined as the time from the week RC/S/W was >50% of peak to the week of the peak. Influenza epidemics usually show an elevated incidence of ILI before the peak and for some weeks after each epidemic. In our study, sharp increases in ILI cases were seen in the weeks before the epidemic; we focused on these weeks. The means for the two parameters across prefectures were calculated for each season. The third parameter was nationwide peak-duration, defined as the time between the first and last week that showed the greatest number of ILI cases among 46 prefectures in each season. Spearman's correlation coefficient was used to analyze the relationship between all pairs of the three indexes. All calculations were performed with Microsoft Excel 2002 (Microsoft Corp., Redmond, WA), and significance was determined at $p < 0.05$.

Results

Influenza Epidemics, 1992–1999

We analyzed 2,586,272 ILI cases during the 7-year period from 1992 to 1999. The annual influenza season began between November and December, peaked between January and February, and returned to baseline between April and June for the study period in every year at all reporting sites (Figure 1). Seasonal peaks in ILI activity occurred annually in all prefectures. Nationwide epidemics lasted for 3–4 months, but successive or overlapping waves of infection by influenza A and B sometimes resulted in a more prolonged outbreak, as in the 1996–1997 season.

The predominant circulating strain was A/H3N2 in all seasons, except in 1996, when it was A/H1N1. Larger scale epidemics were observed in the 1992–93, 1994–95, and 1997–98 seasons, when new antigenic variants of A/H3N2 as predominant circulating subtypes were isolated, namely A/Kitakyushu/159/93, A/Wuhan/359/95, and A/Sydney/5/97, respectively. A relatively large-scale epidemic was also observed in the 1998–99 season, but the predominant circulating subtype was A/Sydney/5/97, as in the previous year. The first peak arose from one predominant viral agent and was bigger than the second peak in the 1996–97 season, with a bimodal curve.

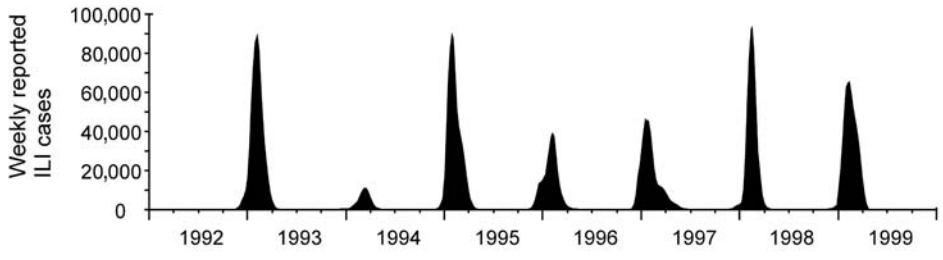

Figure 1. Moving averages of weekly reported influenzalike illness (ILI) cases.

Geographic Analysis

Kriging analysis clearly illustrated spatiotemporal movement of ILI epidemics in Japan (Figure 2). Seasonal ILI activity occurred in a sequential manner, and differences between seasons were easy to identify and characterize. The starting prefectures or areas of the peak ILI activity were mostly in the western part of Japan, except in the 1996–97 season. Trends did not change with the appearance of new variants. The most dramatic differences from year to year were in spreading pattern, as shown in the contour map of peak ILI activity by week. With the monotonous spreading pattern, peak ILI activity covered Japan within 3 to 5 weeks in large epidemics with new antigenic variants of A/H3N2, such as occurred in the 1992–93, 1994–95, 1997–98, and 1998–99 seasons. On the other hand, with the multitonous patterns, peak ILI activity covered Japan within 12 to 15 weeks in small epidemics without new antigenic variants of A/H3N2 in the other four seasons.

Statistical Analysis

During the 7-year study period, the greatest number of ILI cases, the increasing-to-peak period, and the nationwide peak duration were 4.67–40.88 ILI cases per sentinel per week, 3.43–4.83 weeks, and 3–15 weeks, respectively (Figure 3). With the larger epidemics, such as in 1992–93, 1994–95, and1997–98, and, to a lesser extent, 1998–99, the greatest number of ILI cases was >28 RC/S/W, the increasing-to-peak period was <4 weeks, and the nationwide peak duration was <5 weeks. These three parameters were interrelated ($p < 0.05$).

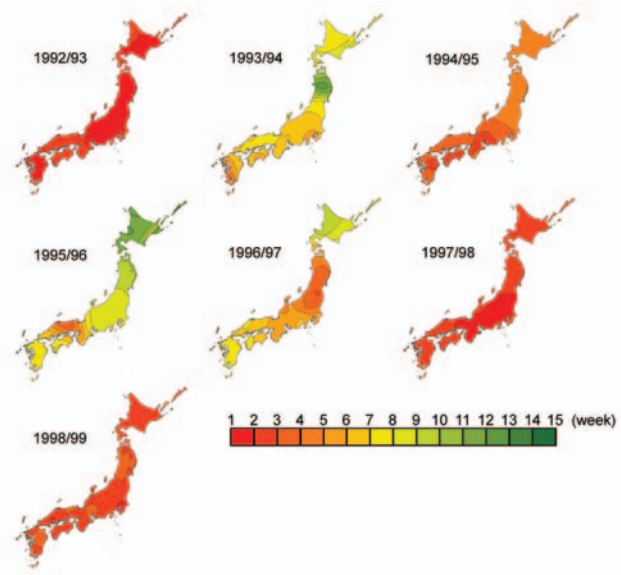

Figure 2. Timing of peak influenzalike illness epidemic activity by week in Japan. The isobars on the contour maps represent interpolated time of peak activity distributed spatially at 1-week intervals. The first week was defined when the peak week was observed first in any one of the prefectures in each season, and then the following weeks were numbered.

Discussion

The size of epidemics and their relative effect reflect interplay between antigenic variation of the virus, protective immunity in the population, and relative virulence of the viruses. Kriging analysis showed several temporal and spatial patterns of influenza epidemics in Japan, which had not previously been clearly recognized.

Climate conditions, especially temperature, strongly affect influenza epidemics. Influenza in temperate areas is characterized by one annual epidemic in winter (4,13,14), and the influenza season occurs from November through April in Japan. The kriging map showed that the first epidemic areas with the greatest number of ILI cases in 46 prefectures were in western-central Japan during the 7-year study period, except in one season. The map showed nationwide epidemic patterns spreading in concentric circles from western-central Japan to eastern Japan. Mean temperature in winter is lower in eastern Japan than in western-central Japan, so cool temperatures are not essential to initiate epidemics.

Immunization coverage, increase in population density, and more frequent international and domestic traffic may have changed the course of epidemics and modified space-time spread (15–17). Immunization coverage is almost the same in all regions of Japan, while population density and traffic are higher in western-central Japan than in the eastern areas. Therefore, we can conclude that the last two factors may affect the nationwide spreading patterns of epidemics (15–17).

The kriging maps showed seasonal ILI activity occurring with two different patterns of peak ILI activity. Larger epidemics with new A/H3N2 variants as antigenic drift showed monotonous patterns, and these epidemics' peak ILI required only 3–5 weeks to cover the whole country. By contrast, small epidemics without new variants showed multitonous patterns, and peak ILI required 11–15 weeks to spread. A relatively large-scale epidemic with A/Sydney/5/97 was observed in the 1998–99 season, as in the previous year. The age distribution of ILI cases in the 1997–98 season was mostly <10 years of age, and in 1998 to 1999, the age distribution was mostly >15 years (18); these became two successive, large-scale epidemics. We conclude that kriging maps can indicate the spreading mode and velocity in conjunction with the extent of antigenic change of A/H3N2. However, the time period used for this analysis may not be representative of influenza over the long term, since we studied a period with an unusual predominance of influenza A/H3N2 viruses. Thus, we need further GIS study to know the spreading mode and velocity in conjunction with various strains.

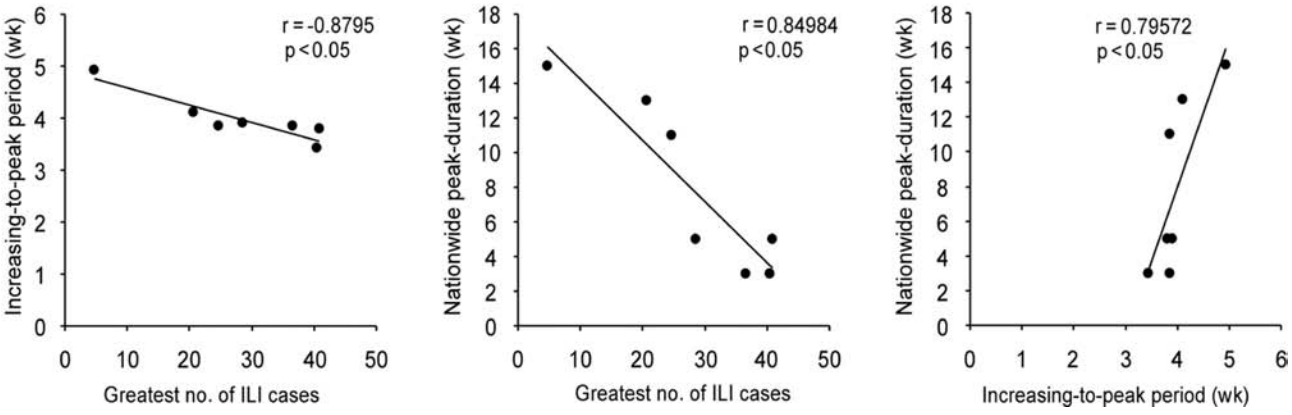

Figure 3. Correlation analysis among three parameters. Greatest number of cases refers to the greatest number of reported influenza-like illness (ILI) cases per sentinel per week (RC/S/W) in each prefecture in each season. The increasing-to-peak period refers to the period from the week when the number of RC/S/W reached >50% of the peak to the peak week. The means of the above two parameters were calculated by season and used for analysis. The nationwide peak-duration refers to the time between the first and last peak week observed among 46 prefectures.

The kriging map allowed us to better visualize and understand spatiotemporal trends in seasonal influenza activity (8,9). To confirm the GIS observations, especially the scale of epidemics and velocities, we developed three parameters: greatest number of ILI cases, increasing-to-peak period, and nationwide peak duration, which demonstrated significant interrelation ($p < 0.05$). We conclude that the larger the greatest number of RC/S/W found, the shorter the increasing-to-peak period and also the shorter the nationwide peak duration. As the scale of the greatest number of ILI cases obtained at the national level was connected with those from prefectural data and had an effect on the spreading mode and velocity of peak ILI activity, the greatest number of ILI cases obtained from the first prefecture in the season also is worthy of attention.

Influenza pandemics occur when a novel influenza virus emerges and most of the world's population has no immunity against it. These pandemics have been observed only with influenza A viruses, which exist in nature as a number of antigenically distinct subtypes and are due to the emergence of a novel hemagglutinin on the virus surface with or without a concomitant change in neuraminidase. In a pandemic, the number of new general practice visits for ILI can be expected to exceed 500 per 100,000 population per week; a medical practice of 10,000 patients would therefore expect to see at least 50 new patients per week (3). Under these conditions, our results indicate that the nationwide peak-duration might be <2 weeks. Therefore, once a pandemic begins, it will be too late to accomplish many key activities required to minimize its impact. Thus, preparatory activities must start well in advance (19). Stockpiling antiinfluenza drugs (1,14,20) seems a reasonable option until prophylactic strategies based on better vaccines can be implemented. Our results demonstrate that GIS is an effective surveillance tool to clarify the dynamics of influenza epidemics.

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Epidemiology and Cost of Nosocomial Gastroenteritis, Avon, England, 2002–2003

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Healthcare-associated outbreaks of gastroenteritis are an increasingly recognized problem, but detailed knowledge of the epidemiology of these events is lacking. We actively monitored three hospital systems in England for outbreaks of gastroenteritis in 2002 to 2003. A total of 2,154 patients (2.21 cases/1,000 hospital-days) and 1,360 healthcare staff (0.47 cases/1,000 hospital-days) were affected in 227 unit outbreaks (1.33 outbreaks/unit-year). Norovirus, detected in 63% of outbreaks, was the predominant etiologic agent. Restricting new admissions to affected units resulted in 5,443 lost bed-days. The cost of bed-days lost plus staff absence was calculated to be £635,000 (U.S.$ 1.01 million) per 1,000 beds. By our extrapolation, gastroenteritis outbreaks likely cost the English National Health Service £115 million (U.S.$ 184 million) in 2002 to 2003. Outbreaks were contained faster (7.9 vs. 15.4 days, p = 0.0023) when units were rapidly closed to new admissions (<4 days). Implementing control measures rapidly may be effective in controlling outbreaks.

Nosocomial gastroenteritis outbreaks, particularly those caused by noroviruses, have become increasingly important in Europe (1) and North America and have attracted media interest (2–4). However, unlike the case of bloodstream, surgical-site, respiratory, skin, and urinary tract infections, tools for detecting and measuring hospital-associated gastroenteritis outbreaks have not been well developed. Accurate measurement of incidence and cost of these infections is precluded (5–8).

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In England and Wales, the Health Protection Agency Communicable Disease Surveillance Centre has operated a passive surveillance system for gastroenteritis outbreaks. From 1992 to 2000, information was collected on >5,000 outbreaks, 27% of which occurred in hospitals and 28% in residential facilities, primarily nursing homes (1,3). Of these outbreaks, >50% were caused by norovirus, and 25% were presumed viral on the basis of clinical signs and symptoms and outbreak characteristics, including high frequency of vomiting, short duration of illness, and short incubation period (1). Particular patterns of transmission have been observed in the outbreaks in healthcare facilities, in which economic effects are likely to be considerable (3).

Because noroviruses are the most common cause of gastroenteritis in the community (9), keeping the virus from being introduced into healthcare settings is difficult, particularly in winter months. For this reason, control measures focus on minimizing the spread of virus within and between hospital units (10). Closing a unit to new patient admissions, excluding affected staff from work for 48 hours postrecovery, and rigorous disinfection are the key features of current control guidelines.

Studies have reported that annually 20%–25% of the population has gastroenteritis (11); however, these surveys excluded persons in healthcare facilities (9). Although many hospital outbreaks of gastroenteritis have been described (12–14), information from systematic, population-based surveillance of gastroenteritis in healthcare settings is lacking (10).

We performed active surveillance of hospital outbreaks of gastroenteritis to determine incidence, microbiologic cause, economic cost, and effectiveness of control measures in the county of Avon, England, an area likely to be broadly representative of England as a whole.

Methods

Surveillance System

Clinical Definitions

Since this surveillance system is designed for detecting outbreaks of gastroenteritis, a two-tiered definition (of cases and outbreaks) was required (Figure 1). These definitions, which draw on Kaplan's criteria of an outbreak of viral gastroenteritis (15), were developed in consultation with public health professionals at all levels of infection control. Ethical approval for this work was obtained from the South West Multi-centre Research Ethics Committee.

Study Population

Gastroenteritis, particularly of viral etiology, is inconsistently reported (16). The county of Avon, England, was selected to focus efforts on collecting complete, high-quality data. The all-cause, age-standardized death rate, and deprivation measures and age distribution indicate that the population of Avon is very similar to that of the whole of England and Wales (http://www.avon.nhs.uk).

Three National Health Service administrations (known as NHS Trusts), comprising four major acute hospitals (similar to secondary/tertiary hospitals in the United States) and 11 smaller community hospitals (similar to primary-level hospitals in the United States) that operate in the sentinel area, were monitored under the surveillance network. Combined, these hospitals have 2,900 inpatient beds, which, on average, maintain 95.6% occupancy of their acute-care beds. In total, 171 "functional care units" were monitored; these units were defined as a room, area or ward regarded as a self-contained area that monitored 171 inpatients. The median number of beds on an inpatient unit was 20 (range 1–38), which reflects the large size of units in NHS hospitals compared to those in many other European or North American designs.

Nursing, medical, and other staff members were included in the population at risk. Time-at-risk for staff members was collected from whole-time equivalent staffing levels supplied by human resources departments. Time-at-risk for patients was calculated by using bed occupancy data from the administration system.

Surveillance and Outbreak Investigation

Each NHS Trust has an infection control team that includes a medically trained microbiologist, a senior infection control nurse, and a team of dedicated infection control nurses. A total of 11 infection control nurses worked at the three trusts. Infection control nurses were responsible for monitoring the populations in their hospitals. Infection control nurses became aware of outbreaks during ward rounds or were alerted to incidents by nurses working on wards. When an event occurred that met the definition of an outbreak (Figure 1), institutions were requested to contact the study coordinator at the Health Protection Agency in Colindale, London. The study coordinator was responsible for ensuring completeness of reports, overseeing data entry, and performing analyses. The study coordinator also solicited monthly null reports in months that no outbreaks were reported in order to confirm that no outbreaks occurred.

Sampling and Diagnostics

Staff members who managed outbreaks were asked to take specimens from the first 10 patients in an outbreak for virologic analysis and from the first 3 patients for bacterial analysis. Such a large number was suggested because of the low sensitivity of viral diagnostics (17). Fecal specimens were preferred, but vomit samples were also accepted for virologic testing. Explicit instructions, based on the Health Protection Agency standard operating procedure (18–19), about taking and sending the samples, were provided. Specimens were tested for viral pathogens at the regional public health laboratory. Specimens were first screened with an in-house enzyme-linked immunosorbent assay (ELISA), followed by reverse transcription–polymerase chain reaction (RT-PCR) for detection of norovirus (20,21).

Outbreak Data

Case forms and an outbreak summary form were completed by infection control nurses as an outbreak pro-

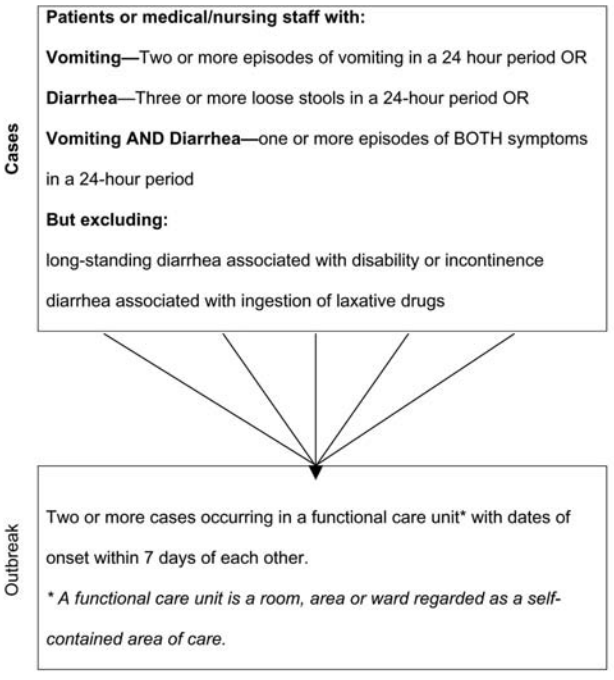

Figure 1. Definition of an outbreak of gastroenteritis in healthcare settings.

gressed. Forms were returned by mail shortly after an outbreak ended. The duration of an outbreak was calculated as the number of days from the onset of the first case to the onset of the last case.

Statistical Analysis

Data were entered and stored in an Access (Microsoft, Redmond, WA) database. Analyses were performed on Microsoft Excel and Stata 8.0 (22). The *t*test was used to compare means; the χ^2 test was used to compare proportions. Continuous data were analyzed with linear regression. Spearman rank test was used to assess correlation of seasonal patterns.

Economic Analysis

The National Health Service of England is a socialized healthcare system. Funding originates from taxpayer money and is distributed by the Department of Health. Resources are allocated to Primary Care Trusts, which commission hospital services from NHS Hospital Trusts (23). Allocations are based on the age distribution of the population served by the hospitals, with adjustments made for maternal needs, mental health, and ambulatory needs of the population (24). Thus, funding is not directly based on the services provided. If healthcare provision is disrupted by an avoidable event, such as hospital-acquired infection, the allocated resources are not used optimally. In other words, opportunity costs (the difference between actual performance of an investment and the optimum expected outcome) are incurred.

We analyzed the opportunity costs of nosocomial gastroenteritis outbreaks to the healthcare service and lost productivity of patients (and the families of pediatric patients). Bed-day loss from new admission restriction for affected units and staff absence from illness were estimated as the two main costs related to gastroenteritis outbreaks in hospitals. Other possible economic effects may include cancelled operations, overuse of beds caused by delayed discharge, additional cleaning procedures, and increased drug prescribing. However, these costs are probably limited, since the illness is relatively short-lived, cleaning is a relatively minor expense, and no treatment is available for viral gastroenteritis except rehydration. We also estimated the societal cost of lost productivity from missed days of work. Intangible costs, such as pain and psychological distress from delayed or cancelled operations and admissions, which are difficult to quantify (25), were not calculated.

Figures from the Unit Costs of Health and Social Care 2002 report were used to estimate the economic loss from empty beds and staff absence (26). Average wage estimates for England were obtained from the Office of National Statistics (27). All costs are in Great British pounds (2002) and converted to U.S. dollars at the rate of £1: $1.6, based on the 5-year average 1999–2003 (http://www.forexdirectory.net/home.html).

For economic estimates, the following assumptions were made. Staff members were, on average, grade E nurses, the mid-range of NHS nursing staff. This figure is probably an underestimate of cost since medical staff, who have higher wages, were also affected in outbreaks. A lost bed-day is a real economic loss in terms of opportunity cost. Since these trusts operate at >95% occupancy of inpatient beds, the result is bed-days lost because the bed would likely have been used. These expenditures cannot be reallocated since infection control guidelines stipulate that staff members from affected units are not to work on unaffected units, and patients from affected units are not to be transferred to unaffected units (10).

Patients of working age (18–64 for men and 18–59 for women) and one family member of each pediatric patient (<18 years) were assumed to be economically active. We assumed that 5 of 7 days of work were missed for each day of illness in these categories. This figure is an overestimate since many days of hospital-acquired gastroenteritis illness would have been spent in the hospital whether the person acquired gastroenteritis or not. Individual length-of-stay data were not available. Hospital staff absence was considered a cost to the healthcare sector, rather than society.

Results

Outbreaks, Cases, and Incidence

In the 171 inpatient units followed, a total of 227 outbreaks occurred; the outbreak incidence was 1.33 outbreaks per unit-year of risk (95% confidence interval [CI] 1.16–1.51) (Figures 2 and 3). All enrolled hospital trusts were affected by outbreaks. Hospital outbreaks peaked in November, with 46 affected units. A smaller peak occurred July, with 22 outbreaks.

Within the 227 outbreaks, 2,154 hospital patients and 1,360 hospital staff met the case definition. The incidence among patients was 2.21 cases per 1,000 hospital-days at risk (95% CI 2.16–2.25) and among staff was 0.47 cases per 1,000 hospital-days at risk (95% CI 0.45–0.50). Units with outbreaks were significantly larger than those that did not have an outbreak in the study period (21.4 vs. 12.6, p value < 0.0001, *t*test).

Diagnostic Results

Specimens were taken for diagnostic analyses in 122 (51%) of the 227 hospital unit outbreaks (Table 1). Norovirus was the confirmed etiologic agent in 61 outbreaks (50%) and was detected in a single specimen in 16 outbreaks (13%). The second most prevalent organism was *Clostridium difficile*, which was confirmed in nine

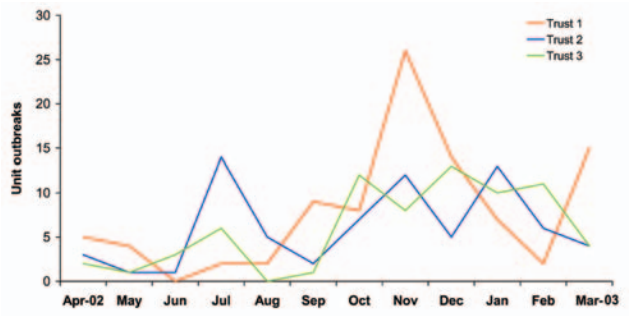

Figure 2. Monthly outbreaks of gastroenteritis in hospitals, Avon, England, April 2002–March 2003 (N = 227).

outbreaks (7%) and detected in a single sample in eight outbreaks (6.5%). Six outbreaks (4.9%) occurred in which both norovirus and *C. difficile* were detected. Rotavirus and *Campylobacter* outbreaks were also detected. Outbreaks from which all specimens were negative for rotavirus and *Campylobacter* (n = 31, 25%) had a seasonal pattern similar to that of norovirus outbreaks (p = 0.13, Spearman rank test) (Figure 3). The monthly distribution of outbreaks in which no specimens were taken correlated with the monthly distribution of norovirus-confirmed outbreaks.

Attack Rates within Outbreaks

In hospital outbreaks, attack rates among staff members (staff affected/staff working on unit: 19.6%, 95% CI 16.6%–22.7%) were significantly lower than those of patients (patients affected/unit beds: 46.8%, 95% CI 40.9%–52.8%) ($p < 0.001$, *t*test). In outbreaks in which norovirus was the confirmed etiologic agent, attack rates were somewhat higher than all outbreaks at 24.5% for staff (95% CI 17.8%–31.2%) and 53.2% for patients (95% CI 41.5%–65.0%), although not significantly so. Attack rates among staff were not higher in the first outbreak (20.6%; 95% CI 16.4%–25.0%) compared to subsequent outbreaks that occurred in the same unit (21.5%; 95% CI 17.2–25.9) (p = 0.8; t-test).

Closing Units to New Admissions and Bed-Day Loss

One hundred and fifty-eight (69.6%) of the 227 hospital unit outbreaks resulted in the affected unit's being closed to new admissions (Table 2). Outbreaks in which norovirus was detected did not result in unit closure to new admissions more frequently than outbreaks in which diagnostic results were negative (71.3% compared to 70.6%, respectively) (p = 0.9, χ^2 test).

Units were closed for a mean of 9.65 (95% CI 8.5–10.8) days, but in the most extreme example, a unit was closed to new admissions for 48 days because of a single outbreak. On average, 3.57 (95% CI 1.86–5.2) bed-days were lost for every day of unit closure to new admissions, which resulted in an estimated 5,443 bed-days lost from gastroenteritis outbreaks.

Economic Loss

Unit closures to new admissions were distributed among unit type specialties (Table 3). The cost of empty beds to the three hospitals was £1.49 million (U.S.$ 2.24 million) or approximately £480,000 (U.S.$ 768,000) per 1,000 beds.

Costs associated with staff absence were calculated as shown in Table 4. A total of 1,360 infections were in staff members; mean duration of illness was 2.4 days. Hospital staff members were advised not to work for 2 days after recovering from gastrointestinal illness (10). If the staff members work 5 days a week, an estimated 3.14 days of work were missed because of illness [(2.4 days ill + 2 days absence postrecovery) x (5 working days/7 days)]. The cost of one day absence was £113 (U.S.$ 181); therefore, outbreaks cost £482,000 (U.S.$ 771,000) or £156,000 (U.S.$ 249,000) per 1,000 beds. Total cost of bed-day loss and staff absence was £1.97 million (U.S.$ 3.15 million), or £635,000 million (U.S.$ 1.01 million) per 1,000 beds.

A total of 971 days of illness occurred among working age men (433 days), working age women (241 days), and children <18 years of age (297 days). Therefore, 139 (971/5 x 5/7) 5-day work weeks were potentially lost. At £476 (U.S.$ 761) per week, total productivity loss is estimated to be £66,000 (U.S.$ 106,000) or £22,700 (U.S.$ 36,400) per 1,000 beds.

Restricting New Admissions

Information about unit closure was available for 52 (85%) of the 61 norovirus-confirmed outbreaks. Forty-

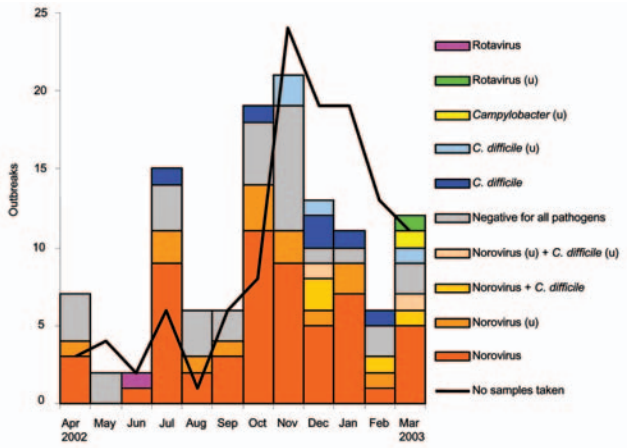

Figure 3. Monthly distribution of outbreaks with diagnostic results (N = 122). Negative outbreaks followed a similar seasonal pattern to norovirus outbreaks. u, unconfirmed (only one positive specimen).

Table 1. Causative organism in hospital gastroenteritis outbreaks, Avon, England, April 2002–March 2003

Organisms	Outbreaks		Combined %
	n	%	
Norovirus[a]	57	46.7	
Norovirus[b]	14	11.5	63.1
Norovirus[a] + *Clostridium difficile*[c]	4	3.3	
Norovirus[b] + *C. difficile*[a]	2	1.6	13.9
C. difficile[b]	6	4.9	
C. difficile[a]	5	4.1	
Rotavirus[a]	1	0.8	
Campylobacter[b]	1	0.8	
Rotavirus[b]	1	0.8	
Negative specimens	31	25.4	
Total with sufficient samples[a]	122		

[a]Confirmed outbreaks (two or more positive specimens). Two or more samples were taken in 122 (54%) of 227 outbreaks.
[b]Unconfirmed outbreaks (single positive specimen).

nine (94%) of these outbreaks resulted in the unit's being closed to new admissions, but only 7 (13.7%) were closed within 3 days of the date of onset of the primary case. Outbreaks in which the affected unit was closed within the first 3 days were contained in a mean 7.9 days (95 % CI 4.3–11.5); outbreaks in units that were not closed or were closed >3 days after the first case lasted for a mean of 15.4 days (95% CI: 13.6–17.3) (p value = 0.002, t-test). Although not reaching levels of statistical significance, the attack rates for patients (0.52 compared to 0.68, p = 0.38), staff members (0.14 compared to 0.27, p = 0.21), and all cases (16.3 compared to 23.7, p = 0.065) all increased if the unit was not shut within 3 days. Units closed within 3 days of outbreak were not different from other units in terms of size, according to linear regression models. Unit size and specialty did not affect the estimated cost of closing the unit to new admissions.

Discussion

In our study, the first published systematic assessment of healthcare-associated gastroenteritis outbreaks (primarily caused by noroviruses), we have demonstrated the cost of such outbreaks. On average, each hospital unit (or ward) had 1.33 outbreaks in the 1-year follow-up period. To control the spread of disease as recommended in national guidelines (10), 158 of the 227 outbreaks resulted in closing the unit to new admissions. This closure resulted in 5,443 lost bed-days, ≈0.5% of all available acute bed-days. This bed loss, combined with staff absence, cost an estimated £635,000 (U.S.$ 1.01 million) per 1,000 beds. The measures taken to control the outbreak are costly, but these data indicate that they may be effective in controlling the duration of an outbreak. Units closed within the first 3 days of an outbreak are contained faster than those not closed or closed after day 4 (7.9 vs. 15.4 days; p = 0.002).

The incidence rates in hospital patients and hospital staff were determined to be 2.21 and 0.47 cases per 1,000 hospital-days of risk, respectively. In other words, a patient who spent a year in the hospital would have an 80% chance of having a case of gastroenteritis during an outbreak. This estimate translates to a 1.5% chance for the average inpatient length of stay (≈7 days). Full-time hospital staff members had a 17% chance of being affected during the year of follow-up. Norovirus was the

Table 2. Characteristics of hospital outbreaks of infectious intestinal disease, Avon, England, April 2002–March 2003[a]

Characteristic	Total
Total inpatient wards followed-up	171
Inpatient unit outbreaks[b]	227
Incidence (outbreaks per unit year) (95% CI)	1.33 (1.16–1.51)
Duration of outbreak	
Mean days per outbreak (95% CI)	9.21 (6.54–11.88)
Unit Closure	
Number of unit closures to new admissions (% of all outbreaks)	158 (69.6)
Total number of days of closure to new admissions	1,527
Mean number of days closed per closure to new admissions (95% CI)	9.65 (8.50–10.81)
Mean number of bed days lost[c] per day of closure to new admissions (95% CI)	3.57 (1.86–5.23)
Total bed days lost[d] (95% CI)	5,443 (2,838–7,968)

[a]CI, confidence interval.
[b]Eleven outbreaks occurred in outpatient units and affected staff members only.
[c]Beds that remained empty because the unit was closed to new admissions. This number does not include beds blocked because patient could not be discharged because of the outbreak.
[d]Days of unit closure to new admissions x bed-days lost per day of unit closure.

Table 3. Hospital unit closure to new admissions and economic loss from empty beds, Avon, England, April 2002–March 2003

Unit	Outbreaks resulting in unit closure to new admissions	Total days of closure	Cost per inpatient bed-day	Total cost (GBP)[a]
Admissions	5	47	273	45,807
Cardiology	11	119	460	195,422
Ear Nose and Throat	1	12	273	11,695
Endocrinology/Diabetes	3	58	273	56,527
Geriatric	26	258	145	133,554
Gynocology	0	2	273	1,949
Intensive Care	1	8	273	7,797
Medical	49	496	273	483,407
Mental Health	5	61	177	38,545
Neurology/Neurosurgery	5	63	272	61,176
Obstetrics/Maternity	1	2	273	1,949
Oncology/Radiology	1	5	354	6,319
Orthopaedic/Ortho Trauma	17	109	273	106,232
Pediatric	4	38	398	53,993
Rehabilitation	2	22	192	15,080
Renal	1	5	273	4,873
Respiratory	8	78	273	76,020
Rheumatology	1	3	241	2,581
Surgery	17	141	368	185,240
Total	158	1,527		£1,488,165 (US$ 2,381,064)

[a]Total days of closure to new admissions x mean days of bed loss per day of closure (3.57 days of bedloss per day of closure) x cost per inpatient bed-day. GBP, Great British pounds.

predominant etiologic agent detected in 63% of hospital unit outbreaks.

The strength of this study and the high quality of data collected were due to the active and systematic approach. The definitions were designed to ascertain outbreaks by using a clear designation of the spatial boundaries. Thus, if infection spread from one unit to another, the events were counted as two separate outbreaks. However, the role of sporadic gastroenteritis in healthcare settings was not assessed in this study. The study team applied standard case and outbreak definitions. Null reporting was used on a monthly basis to confirm that an outbreak had not occurred when none was reported. The full range of modern diagnostics for viral gastroenteritis, including ELISA and RT-PCR assays, was used. However, even using these tests, viral pathogens are not always identified (17). The seasonal pattern of outbreaks in which all specimens were negative in this study suggests that many may also have been caused by noroviruses.

In 2002, norovirus epidemics occurred in the United States, England, Wales, and the rest of Europe (28,29), raising the question: Are the figures reported here representative or the product of an anomalous year? For this reason, this surveillance will continue in forthcoming years to determine whether the cost of the 2002–2003 season is characteristic or not.

Noroviruses are the predominant agent for outbreaks of gastroenteritis in healthcare settings, a finding that is consistent with previous studies from the United States (4), Sweden (30), and historical surveillance data from England and Wales (1,3). However, our study extends these etiologic studies by determining the economic cost of gastroenteritis outbreaks and incidence rates in a defined population. The rates of infection were highest in children and the elderly (31). We could not assess whether length of stay was directly affected by hospital-acquired gastroenteritis or whether death rates increased, since these data were not available.

At U.S.$ 1 million per 1,000 beds, the direct costs to the health sector are substantial and outweigh the indirect cost of lost productivity (overestimated to be U.S.$ 36,400 per 1,000 beds.) This proportional cost to health service is largely because of the age distribution of hospital populations that were affected: <20% of patients were pediatric patients or economically active. Other healthcare sector costs, such as cancelled operations, bed blocking from delayed discharge, additional cleaning procedures, and increased drug prescribing, could be estimated in future studies. However, these costs are probably limited since the illness is relatively short-lived, cleaning is a relatively minor expense, and viral gastroenteritis has no treatment except rehydration. For these reasons, the indirect cost of nosocomial gastroenteritis outbreaks in the community at large are probably small compared to the indirect cost of other hospital-acquired infections that can require postdischarge treatment. However, these results demonstrate the direct effect of nosocomial gastroenteritis outbreaks on healthcare.

Table 4. Costs associated with staff absence from nosocomial outbreaks of infectious intestinal disease, Avon, England, April 2002–March 2003

Row	Item	Figure
A	Number of staff cases	1,360
B	Mean duration of illness	2.4
C	Recommended days staff should remain absent following recovery	2
D	Weekly proportion of days worked	5:7
E	Daily cost of NHS nurse[a]	£113
	Total cost of staff absence[b]	£482,944 (US$ 794,110)

[a]National average cost based on midpoint of grade E nurse.
[b]A x (B + C) x D x E.

"Cost per bed-day" is a broad measure derived from the total net revenue expenditure divided by the total number of inpatient days (26). Thus, cost per bed-day includes overhead expenses, medical and nursing staff payroll, and equipment and treatment costs. U.K. guidelines stipulate that staff members or patients (ill or well) from affected units are to be excluded from unaffected units (10). In other words, as a measure to control further spread, reallocation of resources is prohibited by the guidelines. This restriction can be seen in terms of opportunity cost or a reduction from maximum efficiency. For example, although staff members who are not ill remain at work, they cannot be assigned to other units. Thus, when beds become empty, new admissions are restricted, and nursing services will not be used at maximum efficiency. The quantity of care will decrease, and the resources allocated towards such care will not decline.

Hospital-acquired infections have been estimated to cost the NHS £930 million (U.S. $1,488 million) annually (32). If these costs are distributed evenly across the NHS, hospital-acquired infections would cost the three NHS Trusts (1.7% of all beds in the United Kingdom) in this study £16 million (U.S.$ 25.6 million). Our study suggests that gastroenteritis outbreaks account for 12.5% of that cost, and similar to urinary tract infections, are the most costly healthcare-acquired infection to NHS (32), costing £115 million (U.S.$ 184 million) or ≈1% of the total inpatient services budget. Attributing costs to healthcare-acquired infections is complex, particularly in the case of gastroenteritis outbreaks, because little of the added expense will go directly toward the affected patient.

Not admitting susceptible patients is an effective means of containing nosocomial norovirus outbreaks. Recognizing outbreaks of viral gastroenteritis in hospitals can be difficult because of the high frequency of incontinence and other causes of gastroenteritis, such as antimicrobial-associated diarrhea. ELISA diagnostic kits facilitate rapid diagnosis of norovirus infections (17). Hospital infection control teams should be encouraged to take fecal samples from patients with suspected cases of viral gastroenteritis and to seek diagnoses. A positive confirmation of norovirus should result in immediate restriction of new admissions to the affected unit. Our data suggest that this restriction can be achieved within 3 days of diagnosis of the first case, approximately 1 week of the outbreak's duration can be prevented.

High levels of bed occupancy, the large size of care units, and lack of isolation units in NHS hospitals may make them particularly vulnerable to norovirus outbreaks. Cohorting affected patients is difficult in English hospitals. Units are large (median 20 beds, in this study) and occupancy is very high (>95%), so patients are not easily maneuvered. Policies, procedures, and building design may have major effects on transmission of these infections and should be explored by epidemic modeling, institutional clinical trials, and international studies that analyze the effect of the environment. Thus, our cost estimates are specific to the context of the English NHS. A hospital system that operated at a lower bed-occupancy level or those with smaller numbers of beds per unit may be able to provide nursing care to affected patients, with minimal effect on occupancy or other hospital processes. Economic analyses should be specifically tailored to the healthcare system that is being assessed.

Previous studies on the cost of hospital-acquired infections may have underestimated the effect of gastrointestinal infections because surveillance methods for such outbreaks have historically been lacking. This analysis of an active, enhanced surveillance scheme of three major hospital administrations in a defined geographic area quantifies the cost of gastroenteritis outbreaks to the health service, the important etiologic role of noroviruses, and the positive effect of control efforts.

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Fatal *Naegleria fowleri* Meningoencephalitis, Italy

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We report the first case of primary amebic meningoencephalitis in Italy, in a 9-year-old boy. Clinical course was fulminant, and diagnosis was made by identifying amebas in stained brain sections and by indirect immunofluorescence analysis. *Naegleria fowleri* was characterized as genotype I on the basis of polymerase chain reaction test results.

Primary amebic meningoencephalitis (PAM) is invariably an acute, often fulminant infection caused by *Naegleria fowleri*, a small, free-living ameba that occasionally infects humans and other mammals. Although rare (≈200 cases have been reported worldwide to date), PAM is frequently fatal, is difficult to diagnose, and does not have effective therapeutic options (1–5). Although more than half of PAM cases have occurred in the United States, infections have been registered in countries in every continent. In the past, cases of PAM were reported from Europe, especially from the Czech Republic, Belgium, and the United Kingdom (1). A single case of fatal opportunistic *Acanthamoeba* encephalitis in a patient with AIDS was documented in Italy in 1992 (6). We report the first case of PAM from Italy, diagnosed postmortem in an immunocompetent child who, most likely, acquired the infection in July 2003 after swimming in a polluted water hole of the Po River.

The Case

A 9-year-old boy was admitted to a hospital in Este, a small town in the Veneto region (northern Italy), with a 1-day history of fever and persistent headache on the right side. The child swam and played in a small swimming hole associated with the Po River in northern Italy 10 days before the onset of symptoms. At the time, the region was experiencing an unusually hot summer. On hospital admission, the patient was febrile (temperature 38°C), with a total leukocyte count of 13,780/mm^3 and C-reactive protein level of 1.2 mg/L. No meningeal signs were present on physical examination, and results of a cranial computed tomographic (CT) scan without contrast were normal. On day 2, a stiff neck developed, and the patient became progressively sleepy. A lumbar puncture showed cloudy cerebrospinal fluid (CSF) with 2.5 mmol/L glucose, 4.54 g/L protein, and a leukocyte count of 6,800/mm^3 with 90% neutrophils. Gram-stained CSF smears showed no bacteria, and CSF cultures were negative for bacteria and fungi. On day 3, a blood analysis showed a total leukocyte count 19,600/mm^3 with 91% neutrophils and a C-reactive protein level of 10.6 mg/L. Empiric therapy with ceftriaxone and corticosteroids was started, and the patient was transferred to the intensive care unit of the pediatric department of Padua University Hospital.

Upon admission the child was lethargic, and neurologic evaluation determined a Glasgow Coma Scale score of 9. Treatment with acyclovir and mannitol was started (0.35 g/kg every 6 h). After a few hours, the child became unresponsive to painful stimulation, and he was intubated and mechanically ventilated. Electroencephalogram (EEG) showed decreased electric activity with short, focal, convulsive seizures. Blood and CSF cultures for viruses, bacteria, and fungi were negative, but CSF cell count showed an increase in neutrophils (6,120/mm^3). The next day, arterial hypertension and tachycardia developed in the patient. A repeat CT scan showed a lesion in the right frontal lobe and diffuse cerebral edema (Figure 1A). Approximately 1 hour later, severe anisocoria (10 mm right and 7 mm left) developed, followed by fixed mydriasis. EEG showed isoelectric activity, and the patient was pronounced dead 6 days after onset of symptoms.

Autopsy was performed 30 hours postmortem after obtaining permission from the parents. Body development was normal, and no chronic diseases were evident. The gastrointestinal tract, liver, and other abdominal viscera showed no abnormalities. Gross neuropathologic findings consisted of swollen and edematous brain with opaque and congested leptomeninges. A cerebellar tonsillar herniation and soft, easily breakable frontal lobes were found. Coronal sections of cerebral hemispheres showed diffuse and multiple foci of hemorrhagic necrosis in both gray and white matter. A preliminary histopathologic examination showed a massive and diffuse inflammatory infiltrate, characterized by a high number of neutrophils, few eosinophils or macrophages, and numerous large clusters of cells that morphologically resembled amebic vegetative forms, tentatively classified as *Entamoeba* or *Naegleria*.

When the case was referred to the parasitology laboratory (Infectious Diseases Department, Pavia University Hospital), a definitive diagnosis of PAM was made on the

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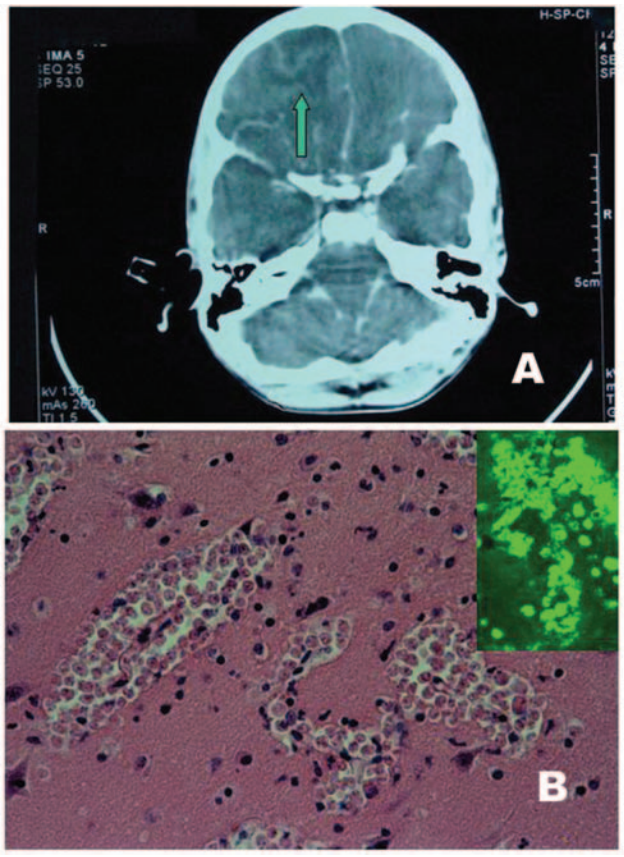

Figure 1. A) Computed tomographic scan: note the right frontobasal collection (arrow) with a midline shift right to left. B) Brain histology: three large clusters of amebic vegetative forms are seen (H-E stain, x 250). Inset: Positive indirect immunofluorescent analysis on tissue section with anti– *Naegleria fowleri* serum.

basis of morphology of amebic trophozoites, which exhibited a conspicuous karyosome, a vacuolated cytoplasm, and a mean diameter of 10 µm to 12 µm. Amebic trophozoites were present in high numbers, often located in perivascular spaces (Figure 1B), and cysts were conspicuously absent, a characteristic feature of PAM.

Formalin-fixed, paraffin-embedded slides and frozen brain specimens were also sent to the Centers for Disease Control and Prevention (Atlanta, GA) for final identification and characterization of the species. Based on the reactivity of amebas in tissue sections with anti–*Naegleria fowleri* serum in indirect immunofluorescent (IIF) analysis (Figure 1B, inset), the etiologic agent was identified as *N. fowleri*.

DNA was extracted from frozen brain tissue. The infected brain tissue was initially subjected to alkaline digestion with 66.6 µL of 1 mol KOH and 18.6 µL of 1 mol dithiothreitol at 65°C for 15 min, neutralized with 8.6 µL of 25% HCl (vol/vol), then buffered with 160 µL of 2 mol Tris-HCl (pH 8.3). DNA was extracted with 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol) (Invitrogen Inc., Carlsbad, CA) and purified with QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA). A fragment (≈600 bp) of the internal transcribed spacer of the rRNA gene was amplified by using primers NF-ITS-F1 [5′-GAC TTC ATT CGT TCT TGT AGA-3′] and NF-ITS-R1 [5′-CTC TTG CGA GGT CCA GAC-3′] (7). Genotype of *N. fowleri* was determined on the basis of DNA sequencing and sequence comparison with published data (Figure 2). DNA sequencing of the ≈600-bp polymerase chain reaction (PCR) product further showed that the ameba was identical to the previously described genotype I or the widespread variant, which was previously found in France, Hong Kong, and the United States (7).

Conclusions

As the clinical and epidemiologic history demonstrate, our patient contracted PAM caused by *N. fowleri* 10 days after swimming and diving in polluted river water in Italy during the unusually hot summer. He displayed characteristic, though not strictly specific, clinical features of PAM (1,2): 1) hyperacute clinical course; 2) unrelenting signs and symptoms of meningitis and encephalitis, the latter confirmed by CT imaging; 3) high levels of peripheral leukocyte count, mainly polymorphonuclear leukocytes; 4) cloudy CSF with leukocytes, hyperproteinosis, low glucose level, and absence of bacteria and fungi; 5) rapid worsening of disease, leading to death within a week. Gross pathologic and histologic findings confirmed the clinical suspicion.

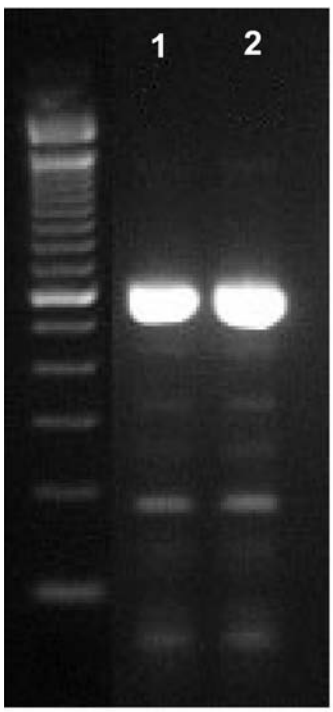

Figure 2. Identification of *Naegleria fowleri* in the brain specimen from the Italian child by polymerase chain reaction analysis of the internal transcribed spacer of the rRNA gene. Lane 1: DNA from infected brain of the patient; lane 2: DNA from CDC:V236 culture, a positive control for *N. fowleri*.

None of the patient's friends and relatives who swam in the same water hole on the same day became ill. Nasal swabs from all of them were negative for amebas, which confirms that fatal *N. fowleri* infection is rare.

Previous epidemiologic studies, conducted in Italy on warm water and thermal mud, failed to isolate *N. fowleri*, although they isolated strains of two other *Naegleria* species, *N. italica* and *N. australiensis*, which are experimentally pathogenic to mice (8–11). Therefore, we initially hypothesized that this case could be caused by one of these species; however, *N. fowleri* was identified by IIF analysis, and PCR confirmed it as genotype I.

This case is the first diagnosed occurrence of PAM in Italy. Few clinicians and microbiologists in Italy are aware of the disease and the potential danger presented by other free-living, pathogenic species of amebas, such as *Acanthamoeba* and *Balamuthia*. Consequently, other cases may have gone undiagnosed.

We emphasize that environmental conditions, in particular, the unusually hot summer of 2003 in Italy and other European countries, have strongly contributed to increasing the surface temperature of natural, open-air basins, such as rivers, lakes, and ponds. According to the forecast by a United Nations scientific advisory panel, global temperature will rise 0.8°C–3.5°C by the year 2100 if production of greenhouse gases is not reduced. An increase in surface temperature will create ideal niches for the thermophilic *N. fowleri* (1,2). Persons who bathe, swim, or dive in pools or freshwater natural basins will increase their chances of coming into contact with *N. fowleri* and contracting PAM.

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Scrub Typhus in the Republic of Palau, Micronesia

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In October 2001, an outbreak of febrile illness began in the southwest islands group of the Republic of Palau. Through October 2003, a total of 15 southwest islanders experienced fever >39.5°C and abdominal distress, both lasting >7days. *Orientia tsutsugamushi*, the agent of scrub typhus, was subsequently identified as the cause.

Scrub typhus, a rickettsial disease caused by *Orientia tsutsugamushi*, is spread by biting larval trombiculid mites. Geographically specific foci of scrub typhus are determined by the distribution of vector mites (1). Rodents of the family *Muridae* are also commonly infected with *O. tsutsugamushi*, and detecting antibodies to the organism in rodents provides evidence for human risk of acquiring the infection (1–4).

After an incubation period of 6 to 21 days, the infection manifests as a lengthy (5–36 days if untreated), nonspecific febrile illness, which is sometimes accompanied by gastrointestinal, respiratory, or central nervous system symptoms. Illness can be inapparent or severe. Death is reported to occur in 1% to 30% of untreated cases (5,6). Scrub typhus is endemic in the tropical and subtropical regions of the Asian continent, as well as in Indonesia, the Philippines, parts of Australia, Japan, northern China, the Russian far east, and Korea (3,5,7–12). After several decades of inactivity, the disease has recently been reported in the Torres Strait Islands of northern Australia and in the Maldive Islands (13,14). The disease has not previously been reported in Micronesia.

The Republic of Palau is an island nation in western Micronesia, 7° north of the equator and 900 km east of the Philippines (Figure). The nation comprises a cluster of main islands within a single outer reef, plus a group of low limestone islands that lie 300 km to the southwest (the southwest islands). Four of these southwest islands are inhabited. Numerous bird species also inhabit the islands. Humans, fruit bats, rats, pigs, and cats are the only resident mammals. Drinking water is collected in rain collection tanks. Residents of the islands farm small plots of land that are cleared from the forest. Each island has a dispensary with basic medications. The islands receive periodic visits from small fishing vessels from Indonesia and the Philippines.

From October 2001 to October 2003, 15 patients from the southwest islands required evacuation to the national hospital in the capital of Palau for treatment of a febrile illness, which was notable for temperature >39.5°C; duration >7 days (average, 12 days); and abdominal pain, vomiting, or both. Some patients had diarrhea, respiratory symptoms, headache, myalgia, and laboratory abnormalities as summarized in Table 1. Headache was described as severe, throbbing, and retroorbital. Sometimes headache was accompanied by photophobia. Rash was noted in 7 patients (47%). It typically appeared on day 4 or 5 of illness, first on the trunk, then spreading to the upper limbs, and lasted 1–3 days. Ten male and 5 female patients were affected. Their mean age was 15 years (range 3–58 years). The illness had no clear seasonal pattern. On two occasions, siblings residing together became ill at the same time. Patients did not appear to improve with empiric antimicrobial drug treatment administered in the field, which included ampicillin, cephalosporins, gentamicin, and metronidazole (none of which are known to be active

Figure. The Palau Islands. Map courtesy of The World Factbook, Central Intelligence Agency, 2004. http://www.cia.gov/ cia/publications/factbook/geos/ps.html

*Department of Health Services, Yap State, Colonia, Yap, Federated States of Micronesia; †Ministry of Health, Koror, Republic of Palau; ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and §Pacific Islands Health Officer Association, Agatna, Territory of Guam.

Table 1. Clinical findings among 15 scrub typhus patients in case series, Palau, 2001–2003

Finding	No. (%) of total patients, N = 15	No. (%) of seropositive patients, N = 6
Fever	15 (100)	6 (100)
Abdominal pain	10 (67)	5 (84)
Vomiting	12 (80)	4 (67)
Headache	10 (67)	4 (67)
Rash	7 (47)	1 (16)
Cough/rhinorrhea	6 (40)	2 (33)
Elevated alanine aminotransferase	6 (40)	2 (33)
Proteinuria	4 (27)	1 (16)
Myalgia	3 (20)	2 (33)
Conjunctival suffusion	3 (20)	2 (33)
Leukocytosis	3 (20)	2 (33)
Diarrhea	3 (20)	1 (16)
CSF pleocytosis	2 (13)	0
Visible blood in stool	1 (7)	0

[a]CSF, cerebrospinal fluid.

against *O. tsutsugamushi*). Two patients had abdominal pain that prompted laparotomy; postsurgical diagnoses were mild appendicitis in one and ileitis in the other.

All but 1 of the 15 patients came from a single island, Sonsoral, which has only 40 inhabitants (Figure). The other lived on the island of Tobi (with 35 inhabitants), approximately 100 miles from Sonsoral, and had not left that island for several years before the onset of illness.

Standard cultures and serologic tests (including those for dengue virus, *Leptospira*, and hepatitis virus), available at the national hospital did not indicate the cause of the illness; however, all patients had received antimicrobial drugs before cultures could be taken. In April 2003, paired serum specimens from one patient that were sent to a commercial reference laboratory in Hawaii had negative results for *Salmonella typhi* and *Rickettsia prowazekii*. At the request of the Ministry of Health, the Centers for Disease Control and Prevention (Atlanta, GA) performed serologic testing of specimens collected from six patients. Results were negative or indeterminate for typhoid fever (by Typhidot IgG and IgM and Tubex (Typhidot, Malaysian Biodiagnostic Research SDN BHD, Kuala Lumpur, Malaysia). Each serum sample was tested for antibodies to *O. tsutsugamushi* by indirect immunofluorescence assay (IFA) after the method of Elisberg and Bozeman (15). Antigen suspensions from the Karp strain of *O. tsutsugamushi* were prepared in chicken yolk sac, and vials of antigen suspension at optimal dilution were frozen at –75°C. The antigen suspension was pipetted onto slides coated with bovine serum albumin (BSA, 1%), air dried, fixed with acetone, and stored at –75°C until use. Slides were warmed to room temperature in desiccated conditions. Serial twofold dilutions, beginning at 1/16, were made in sample diluent (phosphate-buffered saline [PBS], pH 7.38 with 1% BSA and 1% normal goat serum). For the initial screening, two dilutions (1/16 and 1/256) were added to slides and incubated for 30 min at 37°C, followed by washing in PBS, pH 7.38, for 15 min (3 washes x 5 min). An optimized dilution (1/150) of fluorescein isothiocyanate (FITC)-labeled goat antihuman conjugate immunoglobulin (Ig) G (γ-chain-specific) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was then applied to the slides, which were incubated and washed as before, except that eriochrome black T counterstain was added to the middle wash. Glycerol-PBS mounting medium was added to each well, a coverslip was applied, and the slides were read at a magnification of 400x with an epifluorescence UV microscope. Any reactive samples were then titrated to endpoint by using IgG-specific (γ) conjugate. Titers were recorded as the reciprocal of the highest dilution displaying specific fluorescence.

For IgM testing, the samples were first depleted of IgG by using a recombinant Protein G device (Rapi-Sep-M kit, Pan Bio, Columbia, MD). This procedure resulted in a final 1/8 dilution of the serum sample. This solution was then diluted further in sample diluent and placed onto slides. The protocol is similar to that detailed above, but it used FITC-labeled, goat antihuman IgM (μ-chain specific) conjugate at a working dilution of 1/100. Only one serum specimen was available for two of the patients at day 10 and day 36 of their illness; paired serum specimens were available for the other four. The serum specimens of all six patients had high titers of antibodies to *O. tsutsugamushi*. (Table 2).

In this outbreak of scrub typhus in the southwest islands of the Republic of Palau, abdominal distress was a prominent feature. However, none of our patients had an inoculation-site eschar, including the two patients who were examined after we became aware of the disease in the southwest islands. The eschar associated with scrub typhus can have minimal symptoms and be hidden within skin folds or hairy body areas. The absence of eschar has been noted previously in Southeast Asian patients (11). Although no deaths occurred in this outbreak, the cases were sufficiently severe to require evacuation by boat, a difficult and expensive measure that is taken only in cases of life-threatening illness in Palau. Sonsoral, the island with 14 of the cases, has a population of 40 residents; thus, the attack rate for symptomatic disease on Sonsoral was 35%, higher than has previously been reported for this disease.

After this cluster of scrub typhus cases was recognized, a campaign to educate the local community about the disease was launched in the southwest islands and in the capitol. Controlling the rat population, wearing clothing and using repellants when in contact with grass and brush, and eliminating brush near households were emphasized. The

Table 2. Exposure factors significantly associated with campyloba poultry[b,c]

Factor
Eating raw, rare or undercooked poultry
Consuming raw milk or raw milk products
Professional exposure to animals or contact with farm or zoo animals
Eating turkey or chicken in a restaurant, a fast food restaurant, or a buffet
Eating smoked turkey or chicken
In a restaurant, a fast food restaurant, or a buffet
At home
Eating poultry cooked in fondue
Eating microwaved poultry
Eating barbecued poultry
Handling raw poultry
Eating microwaved chicken croquettes
Using the same plate to carry raw meat or poultry and to take it back once cooked
Eating turkey or chicken at home
Eating ground turkey or chicken

[a]By univiriate conditional logistic regression for matched data, adjusted for t
[b]Not associated with campylobacteriosis.
[c]OR, odds ratio; CI, confidence interval.

public and healthcare workers are also taught the importance of early recognition and antimicrobial drug treatment of possible patients.

Important questions remain regarding the reasons for the high attack rate on Sonsoral, whether scrub typhus is newly introduced in the region or only newly recognized, and the distribution and dynamics of *O. tsutsugamushi*, its vector, reservoir(s), and human hosts in Palau and elsewhere in Micronesia. Serologic assessments and studies of the local environment are needed to clarify these issues.

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Instructions for Infectious Disease Authors

Dispatches

Articles should be 1,000–1,500 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 two); and a brief biographical sketch of first author—both authors if only two. 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.

Long-term SARS Coronavirus Excretion from Patient Cohort, China

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This study investigated the long-term excretion of severe acute respiratory syndrome–associated coronavirus in sputum and stool specimens from 56 infected patients. The median (range) duration of virus excretion in sputa and stools was 21 (14–52) and 27 (16–126) days, respectively. Coexisting illness or conditions were associated with longer viral excretion in stools.

Severe acute respiratory syndrome (SARS) is a newly emerged disease that spread globally in early 2003; more than 2,523 cases and 181 deaths occurred in Beijing, China (1–3). In the initial outbreak period, the diagnosis of infection was mostly dependent on clinical manifestations and epidemiologic findings, until the associated coronavirus (SARS-CoV) was identified as the causal agent (4–6), and its immediate full genome was sequenced (7,8).

Molecular assays using reverse transcriptase–polymerase chain reaction (RT-PCR) were developed as an important diagnostic tool on a variety of clinical samples (4–6,9). Several studies have explored the optimal source and timing of sample collection for SARS diagnosis on the basis of RT-PCR results (5,10–12). While viral genetic material could be detected by RT-PCR in stools for up to 50 days, no virus could be cultured after 3 weeks of illness from corresponding specimens (12). Although long-term viral excretion has important public health implications through its potential spread of the virus to the environment, no study has yet explored factors associated with prolonged viral excretion and risk for transmission to household contacts. Our study was designed to investigate prospectively the viral shedding from a cohort of Beijing SARS patients for up to 6 months. Clinical and epidemiologic data were used to identify the potential risk factors for prolonged virus shedding. Household contacts of SARS patients were clinically monitored for secondary transmission.

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The Study

Patients were recruited for the study at one of the designated hospitals for SARS patients in Beijing. The diagnosis of probable SARS was made in accordance with the definition promulgated by the World Health Organization. Briefly, the case definition included the following: fever of ≥38°C, cough or shortness of breath, new pulmonary infiltrates on chest radiography, and a history of exposure to a patient with SARS or absence of response to empirical antimicrobial therapy for typical and atypical pneumonia. Stool and sputum specimens from probable SARS patients were collected weekly while they were in the hospital and monthly after they were discharged, up to 6 months after onset of disease. Specimens were transported in viral transport medium and stored at –70°C until testing.

After informed consent was obtained, epidemiologic and clinical data were collected from all study participants by using a standard data collection form, through interview and medical record review. The obtained information included the following items: age, sex, occupation, medical history, time and nature of exposure, symptoms and physical findings, laboratory tests at admission to the hospital, and outcomes on discharge. A questionnaire was also used to identify any suspected or probable SARS case among household contacts of SARS patients after they were discharged. The questionnaire was administered 1 month after discharge from the hospital, and at the end of the followup period.

Specimens were analyzed after the RT-PCR protocol was optimized in the laboratory. Early samples from all patients were tested, and only patients with at least one sample that tested positive for SARS-CoV RNA during the first 16 days were kept in the study. All subsequent samples from the same patients were then tested until three consecutive samples were negative; the first negative sample defined the time of negativation. RNA from clinical samples was extracted by using the QIAamp virus RNA mini kit (Qiagen, Hilden, Germany), as instructed by the manufacturer. Total RNA was then reverse transcribed with random hexamers, and cDNA was amplified with two primer pairs. The first pair, COR-1/COR-2, is one of the primer pairs recommended by the World Health Organization (http://www.who.int/csr/SARS/primers/en/). The second pair, R1/R2, was designed to amplify a 220-bp fragment of the replicase gene (R1: 5′ AGG TTA GCT AAC GAG TGT GCG 3′, R2: 5′AGC CTG TGT TGT

AGA TTG CGG 3′). These two primer pairs, COR-1/COR-2 and R1/R2, had been selected among others (including Cor-p-F2/Cor-p-R1, Cor-p-F3/Cor-p-R1, SAR1s/SAR1as) for their higher sensitivity (87% and 95%, respectively), using serologic testing as the standard. Congruence between the results of the two tests was 86% (F. Tang, pers. comm.). Only specimens with positive results for both primer pairs were considered positive, so that false-positive results were eliminated. During this procedure, positive and negative controls were systematically included and treated in the same way as the virus sample.

Viral isolation was performed on RT-PCR–positive stool samples from convalescent patients only. The sample was pretreated by centrifugation, and the supernatant was injected after filtration into the VeroE6 cell culture at 37°C. All procedures were handled under biosafety level 3 conditions. We looked for the characteristic cytopathic effect for 5 to 11 days after injection. Examination with electron microscope and RT-PCR assays were used for identification of SARS-CoV.

All data were processed by SPSS (SPSS Inc. Chicago, IL) and Stata (Stata Corporation, College Station, TX) software. Categorical and continuous variables were compared across study groups by means of χ^2, Fisher exact test, and Mann-Whitney U tests, where appropriate. Survival analysis was performed by using time to RT-PCR negativation as the outcome variable (for stool and sputum specimens separately). Survival curves were built by using the Kaplan-Meier method. Correlation coefficient between time to RT-PCR negativation in stools and sputum was calculated by using the Spearman rank correlation coefficient.

From mid-March to early May 2003, a total of 83 probable SARS patients were admitted to a Beijing hospital dedicated to SARS patients. Of these, 56 (67.4%) had at least one RT-PCR–positive sample in the first 16 days of hospitalization and were therefore enrolled in the study. The study group consisted of 31 male and 25 female participants, with a median age of 31 and 34 years, respectively. Of the 56 patients, 21 (37.5%) were healthcare workers. The median (range) duration between onset of symptoms and hospitalization was 2 (0–6) days, and the median (range) duration of hospital stay was 32 (21–58) days. Six patients had coexisting medical conditions or illnesses at enrollment or during followup: diabetes (2 patients), heart disease (2 patients), pulmonary tuberculosis (2 patients, including 1 with diabetes), and high blood pressure (1 patient). No patient died.

A total of 514 stool and 493 sputum specimens were collected from the 56 patients during the study period. The median (range) duration between onset of symptoms and first positive RT-PCR test result was 6 (3–10) days for stool and 6 (3–16) days for sputum. RT-PCR sputum and stool specimens did not show negative results until day 14 and day 16 after disease onset, respectively (Figure). The median (range) duration of viral excretion was 21 (14–52) days for sputum samples, and 27 (16–126) days for stool specimens. Negative RT-PCR results occurred at the same time in sputum and stool samples for 45 patients, and at a later time in stool samples than in sputum in 11 patients (the Spearman rank correlation coefficient between time to RT-PCR negativation in sputum and stool was 0.65, $p < 0.0001$). Four patients had viral excretion in stools after 100 days; three of these patients had coexisting conditions, such as pulmonary tuberculosis (2 patients), diabetes (1 patient, who also had tuberculosis), and high blood pressure (1 patient). The proportion of patients with coexisting conditions was significantly higher for patients with viral excretion >100 days compared to others (3/4 vs. 3/52, $p = 0.003$, Fisher exact test). All attempts (n = 12) to isolate virus from RT-PCR–positive stool specimens collected ≥6 weeks after disease onset failed. All 56 patients had at least one close contact. Clinical symptoms suggestive of SARS did not develop in any of the 70 close contacts of convalescent SARS patients we surveyed.

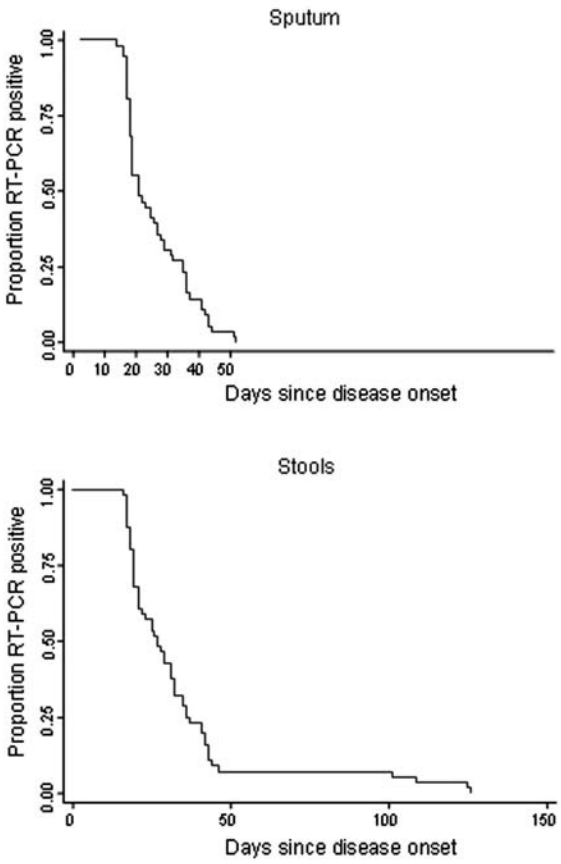

Figure. Time lapse before sputum and stool specimens of patients with severe acute respiratory syndrome (SARS) (N = 56) were negative by reverse transcription-polymerase chain reaction, at one designated SARS hospital, Beijing, 2003.

Conclusions

This study is the first to document duration of SARS-CoV excretion in sputum and stool in a cohort of SARS patients. The median duration of viral excretion was 3 weeks in sputa and 4 weeks in stool samples. This estimate might be high, since some patients with no or short viral excretion might have been missed because of the study design. In most patients (45 of 56), the duration of viral excretion in both specimens was identical, while it was longer in stool samples compared to sputa for 11 patients. The long duration of excretion (>100 days after onset of symptoms) observed in stools of four patients suggests that independent replication of virus may take place in the intestinal tract. Three of these four patients had coexisting conditions, a known risk factor for prolonged SARS illness (13,14). Prolonged viral excretion may have important public health implications if responsible for spread of the virus to other persons or to the environment. Two findings of this study are reassuring in this regard: one is the failure of all attempts to isolate the virus from positive RT-PCR specimens collected in convalescent patients, which suggests that excreted virus was no longer infectious; the other is the absence of SARS in close contacts of convalescent patients during the study period.

Our study has demonstrated long-term duration of SARS-CoV excretion in stools. Careful hand and toilet disinfection is required to avoid transmission of the virus to close contacts of patients.

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Campylobacteriosis, Eastern Townships, Québec

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Independent risk factors for campylobacteriosis (eating raw, rare, or undercooked poultry; consuming raw milk or raw milk products; and eating chicken or turkey in a commercial establishment) account for <50% of cases in Québec. Substantial regional and seasonal variations in campylobacteriosis were not correlated with *Campylobacter* in chickens and suggested environmental sources of infection, such as drinking water.

Published case-control studies provide conflicting results regarding the risk factors for sporadic campylobacteriosis. Poultry is commonly considered the principal source, and in some studies, was implicated in 50% to 70% of endemic cases (1,2). *Campylobacter* have been frequently cultured from poultry during processing (47%–82%) and retail distribution (23%–62%) (3–6). However, some studies observed no significant risk associated with eating chicken (7,8); in other studies, this factor was actually protective (9,10). We describe a prospective case-control study of domestically acquired *Campylobacter* infections combined with a prevalence study of *Campylobacter* spp. in whole retail chickens purchased in the Eastern Townships, Québec.

The Study

The Eastern Townships comprise seven counties and total ≈300,000 inhabitants. Hospital microbiology laboratories routinely report all *Campylobacter* enteritis cases to the regional public health department. All the laboratories in the study region, except in Granit County, routinely evaluated stool specimens for *Campylobacter* by using comparable standard methods for isolation and identification (Karmali or Skirrow media incubated for 72 h at 42°C in a microaerobic atmosphere). Granit County's laboratory sent stool specimens for *Campylobacter* culture to our hospital microbiology laboratory on special medical request only. Incidence rates of campylobacteriosis in the Eastern Townships and Québec Province were calculated with demographic and reportable diseases data from provincial registers.

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All cases reported from July 1, 2000, through September 30, 2001, were eligible. Case-patients were excluded if the infection was acquired outside Québec (i.e., travel abroad during the 10-day period before the onset of symptoms) or if the interval between the onset of symptoms and reporting was >6 weeks. All investigations were conducted within 2 weeks of reporting. For participants with infections reported on multiple occasions during the study period, the first episode of infection was considered. The median interval from the onset of symptoms to the interview of the cases was 13 days (range 5–56 days; 90th percentile, 23 days).

Each case was matched for sex and age group (<1, 1–4, 5–14, 15–34, 35–64, and ≥65 years) to two controls living in the Eastern Townships, who were identified through random digit dialing. Patients and controls were interviewed by telephone with a structured questionnaire to capture demographic and clinical data, travel history, food history, water consumption, recreational water activity, animal contacts, and other illness during the 10 days before the onset of symptoms. Controls had to be interviewed within 3 weeks of the patient and were excluded if they could not be reached after three telephone calls; had fever, abdominal pain, nausea, vomiting, diarrhea, or bloody stools; traveled abroad during the 10-day period before the patient's onset of symptoms; or refused to participate. Controls did not have stool samples tested for *Campylobacter*. A surrogate parent was interviewed when the patient or control was a child <14 years of age. The interviewers were not blinded to the patient or control status of study participants.

Risk factors for campylobacteriosis were evaluated by conditional logistic regression for matched data adjusted for the county of residency. All risk factors with $p < 0.05$ by univariate analysis were included in a multivariate, conditional, logistic regression, stepwise selection model for matched data. All statistical analyses were performed using SAS version 6.1 (SAS, Cary, NC).

During the study, four fresh, eviscerated whole chickens were bought weekly in different counties (one chicken per store); for each county, the number of chickens sampled monthly was proportional to the population. Retail chickens sold in the Eastern Townships are produced by multiple companies based elsewhere in Québec Province.

The chickens were stored at 4°C overnight and washed vigorously with 250 mL of nutrient broth. The broth was filtered through cheesecloth and centrifuged at 16,300 x *g* for 15 min. The sediment was suspended in 5 mL of brucella broth; 100 mL of Park and Sanders' selective enrichment broth with 0.5 mL of Supplement A (0.2% vancomycin and 0.2% trimethoprim lactate) and 5 mL of Supplement B (0.064% sodium cefoperazone in brucella broth) (11) were added to the suspension, gently mixed,

and incubated under microaerobic atmosphere at 37°C for 4 h, then at 42°C for 48 h. Three loopfuls (0.05 mL) of the suspension were plated on Karmali agar and incubated at 42°C for 48 h under microaerobic conditions. Isolates of *Campylobacter* were identified to the species level by routine phenotypic methods.

From July 2000 through October 2001, a total of 201 cases of campylobacteriosis were reported, of which 43 were excluded: 18 patients acquired their infection outside Québec, 18 resided outside the Eastern Townships, 6 could not be interviewed within 6 weeks after the onset of symptoms, and 1 patient declined to participate. All but two patients were matched to two controls each; consequently, the final dataset comprised 158 cases and 314 controls. Cases and controls were well-distributed across the seven counties, except in Val St-François, which represented 15% of cases and 7% of controls (data not shown).

During the study period, the mean crude incidence of campylobacteriosis was 63.1/100,000 in the Eastern Townships, compared to 44.5/100,000 in the remainder of Québec Province ($p < 0.0001$). Most cases occurred during July, August, and September (Figure 1). The median age of the case-patients was 31 years (range 11 days to 91 years). The incidence of campylobacteriosis varied considerably by age (Figure 2), with the highest rates among children 0–4 years of age (169.2/100,000) and young adults 15–34 years of age (mean = 79.4/100,000). Overall, 64 (40.5%) participants were female.

The rates varied from 38.3/100,000 in Memphrémagog to 113.5/100,000 in Asbestos (excluding Granit, where case ascertainment was different); these interregional differences persisted after stratification for age (Table 1). The risk of campylobacteriosis was 2.4-fold higher in Asbestos ($p = 0.0001$) and 1.3-fold higher in Val St-François ($p = 0.04$) than elsewhere in the Eastern Townships.

Among 41 exposure factors evaluated by univariate conditional logistic regression, four achieved p values < 0.01 (Table 2). Two were associated with poultry: eating raw, rare, or undercooked poultry ($p = 0.003$) and eating turkey or chicken in a restaurant, a fast food establishment, or a buffet ($p = 0.004$). Two were associated with other exposures: consuming raw milk or raw milk products ($p = 0.0001$) and professional exposure to animals or a contact with farm or zoo animals ($p = 0.0003$). No other activity related to consuming or handling poultry appeared related to infection (Table 2).

Conditional multivariate analysis adjusted for the county of residency resolved only three independent risk factors: raw, rare, or undercooked poultry (odds ratio [OR] 5.00, 95% confidence interval [CI] 1.79–13.98, $p = 0.002$), raw milk or raw milk products (OR 3.67, 95% CI 1.95–6.90, $p = 0.0001$), and turkey or chicken eaten in a restaurant, a fast food or a buffet (OR 1.96, 95% CI 1.24–3.11, $p = 0.004$). These factors accounted for 8%, 18%, and 20% of cases, respectively.

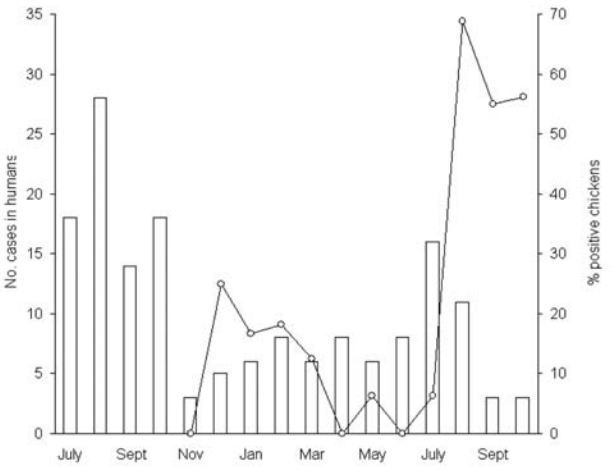

Figure 1. Monthly distribution of the number of sporadic cases of *Campylobacter* infections in humans from July 2000 to October 2001 (columns) and of the prevalence of *Campylobacter* in whole retail chickens from November 2000 to October 2001 (line graph).

A total of 177 chickens from 58 different food stores were cultured (median per month, 16; range 8–20). *Campylobacter* spp. were cultured from 41 (23%) (37 *C. jejuni*; 4 *C. coli)*. The prevalence of *Campylobacter* was low from November 2000 to July 2001 inclusively, with 0–2 positive chickens (0%–25%) per month (Figure 1) but increased sharply in August, September, and October 2001, with rates reaching 69%, 55%, and 56%, respectively. The number of locally acquired *Campylobacter* enteritis in humans peaked at 16 cases in July 2001 (i.e., 1 month before the peak of chicken contamination) and then decreased to 11, 3, and 3 cases in August, September, and October 2001, respectively. Further, we analyzed data for each county separately and found no geographic correlation between campylobacteriosis in humans and

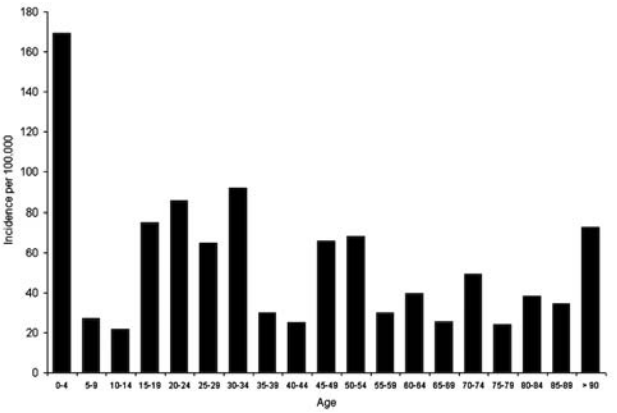

Figure 2. Distribution of the incidence rates of *Campylobacter* infection by age in the Eastern Townships.

Table 1. Incidence rates of campylobacteriosis in each county in the Eastern Townships with crude and age-stratified relative risk compared with incidence rates in the other counties in the region

County	No. of cases	Total population	Incidence rate per 100,000	Crude RR	RR stratified for age[a]	p value[b]
Asbestos	17	14,975	113.5	2.23	2.37	0.0001
Val St-François	23	28,809	79.8	1.48	1.33	0.04
Sherbrooke	85	143,792	59.1	1.09	1.14	NS
Coaticook	9	16,444	54.7	0.97	0.85	NS
Memphrémagog	16	41,785	38.3	0.64	0.79	NS
Haut St-François	10	22,358	44.7	0.78	0.74	NS
Granit	4	21,905	ND[c]	ND[c]	ND[c]	ND[c]

[a]The relative risk (RR) represents the incidence rate of campylobacteriosis in one county compared to the incidence rate in the other counties taken as a whole, before (crude RR) and after stratification for age (stratified RR).
[b]The p values apply to the stratified relative risks.
[c]Rates not calculated for County of Granit because of different case ascertainment process used there; see text for details.

Campylobacter in chickens (p = 0.42). Thus, although chicken consumption is an important risk factor for *Campylobacter* enteritis, it does not explain either the seasonal or regional variations in the incidence of sporadic cases of campylobacteriosis in humans.

Conclusions

Exposures to poultry account for fewer than half the episodes of sporadic *Campylobacter* infection. Substantial seasonal and interregional variations suggest environmental sources of infection. In the univariate analysis, drinking tap water at home or at work tended to be associated with an increased risk for infection (OR 1.90, p = 0.03), and in a subanalysis of cases in Asbestos County, which had the highest incidence, drinking tap water from a deep well at home was the only risk factor identified (53% of cases compared to 23% of controls; OR 3.83, p = 0.06 by univariate analysis and OR 3.96, p = 0.06 after adjusting for age group and sex). A recent case-control study (12) identified drinking water that was not disinfected as an independent risk factor for campylobacteriosis, with an etiologic fraction of 26%. These results are consistent with the hypothesis that the waterborne route of infection may be the common underlying pathway linking infection in humans, poultry, other domestic animals, and wild birds.

In waterborne outbreaks associated with *Campylobacter*, fecal contamination of the drinking water source has been traced to runoff of surface water after rain or to leakage from a sewage line into an adjacent drinking water pipe (13–15). Since a few hundred viable organisms represent an infectious dose, even apparently low levels of contamination could result in infection. The true importance of drinking water as a source of sporadic infection in humans may have been underestimated in the past and should be investigated in future studies.

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We thank Diane Dion, Danielle Proulx, Linda Billard, and Mélanie Proulx for data collection; Reno Proulx for designing the

Table 2. Exposure factors significantly associated with campylobacteriosis[a] and other factors relating to consuming and handling poultry[b,c]

Factor	Case-patients	Controls	OR	95% CI
Eating raw, rare or undercooked poultry	13/154	7/310	4.51	1.67–12.14
Consuming raw milk or raw milk products	33/153	25/310	3.12	1.78–5.48
Professional exposure to animals or contact with farm or zoo animals	39/158	36/312	2.53	1.44–4.13
Eating turkey or chicken in a restaurant, a fast food restaurant, or a buffet	57/140	77/289	1.89	1.23–2.90
Eating smoked turkey or chicken				
In a restaurant, a fast food restaurant, or a buffet	5/156	6/309	1.67	0.50–5.57
At home	42/153	93/310	0.90	0.58–1.38
Eating poultry cooked in fondue	5/156	7/312	1.49	0.46–4.79
Eating microwaved poultry	2/158	3/309	1.36	0.22–8.26
Eating barbecued poultry	34/157	66/310	1.02	0.64–1.64
Handling raw poultry	78/153	160/314	0.97	0.66–1.44
Eating microwaved chicken croquettes	5/157	11/308	0.92	0.31–2.72
Using the same plate to carry raw meat or poultry and to take it back once cooked	38/156	66/302	0.78	0.48–1.29
Eating turkey or chicken at home	128/140	274/289	0.58	0.26–1.27
Eating ground turkey or chicken	3/158	12/314	0.50	0.14–1.79

[a]By univariate conditional logistic regression for matched data adjusted for the county of residency.
[b]Not associated with campylobacteriosis.
[c]OR, odds ratio; CI, confidence interval.

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Laboratory-acquired Brucellosis

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We report two laboratory-acquired *Brucella melitensis* infections that were shown to be epidemiologically related. Blood culture isolates were initially misidentified because of variable Gram stain results, which led to misdiagnoses and subsequent laboratory exposures. Notifying laboratory personnel who unknowingly processed cultures from brucellosis patients is an important preventive measure.

The incidence of brucellosis may reach 200 per 100,000 population in some developing countries, but in the United States brucellosis is a rare disease (1). Over the past 50 years, effective control of brucellosis in animals and animal products in the United States has dramatically reduced the number of infections (2). Because of the rarity of cases, laboratory and medical personnel may not be familiar with *Brucella*, its clinical manifestations, and its laboratory characterization.

Transmission of brucellosis occurs from ingesting, directly contacting, or inhaling the organism. Exposures most commonly occur by eating contaminated animal products from disease-endemic areas (1). Other, less common routes include person-to-person transmission and accidental infection with live animal vaccines (3,4). *Brucella* species are classified as category B bioterrorism threat agents, so use as a bioweapon must also be considered as a potential source of exposure (5).

Laboratory workers are at risk when handling specimens containing *Brucella* species because of aerosol-generating procedures or accidents that may result in infection of blood or conjunctiva (6). In fact, brucellosis is one of the most common laboratory-acquired infections (6–14). To identify *Brucella* spp. from blood cultures, laboratory workers rely on the Gram stain as a preliminary test. *Brucella* organisms are gram-negative coccobacilli and may be present in high concentration in blood cultures drawn early in the disease process. If the Gram stain is misinterpreted, the organism may be misidentified, which would result in misdiagnosis and potential opportunities to expose laboratory personnel (10).

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From 1993 to 2002, five *B. melitensis* and three *B. abortus* infections were reported in New York State, exclusive of New York City. Of the eight reported cases, two were laboratory-acquired, five resulted from ingesting unpasteurized milk products from countries with endemic disease, and one had an undetermined source of infection (New York State Department of Health [NYSDOH], unpub. data). We describe two cases of laboratory-acquired brucellosis in New York State that were not known to be related at the time of their diagnoses. Initial isolates from blood cultures of an index patient and two laboratory workers were incorrectly identified as contaminants, in part because of reporting of primary Gram stain results as gram-positive and gram-variable.

Case Reports

In early November 2001, a 57-year-old female laboratory worker (laboratory worker 1) began experiencing nonspecific symptoms of malaise, vomiting, headache, lower leg cramping, anorexia, and fever. One week after onset of symptoms, she was evaluated for severe headaches at a local emergency room, where cerebrospinal fluid (CSF) and blood cultures were collected. The CSF culture was negative. From the blood culture, small, gram-positive bacilli were isolated and characterized as coryneform bacilli, which are usually interpreted as contaminants of unknown clinical importance. Despite multiple hospital admissions, the laboratory worker continued to have symptoms, but her condition remained undiagnosed. Approximately 5 weeks after symptom onset, colleagues from the hospital microbiology laboratory where she was employed (laboratory 1) drew her blood for culture again. After 5 days of incubation, gram-variable coccobacilli, later identified as *Brucella* spp., were isolated. Subculturing and biochemical tests were conducted in a class II biosafety cabinet. The *Brucella* serum agglutination test (SAT) was reactive (1:640) at the NYSDOH laboratory, Wadsworth Center. Laboratory worker 1 was initially treated with doxycycline and gentamicin, followed by doxycycline and rifampin, for 6 weeks of outpatient therapy. The isolate was later identified as *B. melitensis* by the Wadsworth Center and confirmed by the Centers for Disease Control and Prevention. The patient has not relapsed 18 months after completing treatment.

In a second incident in mid-January 2002, a 48-year-old woman had nocturnal temperature spikes to 40°C, chills, drenching sweats, and weight loss. Initially, she had a diagnosis of influenza and was treated with oseltamivir phosphate. Symptoms persisted, and uveitis developed. In early March 2002, a diffuse, erythematous rash appeared on the anterior aspect of both legs. A blood culture and serologic tests for Lyme disease, ehrlichiosis, and Rocky Mountain spotted fever (RMSF) were performed. From the

blood culture, gram-positive cocci were isolated and identified as *Micrococcus* spp. by a commercial laboratory. RMSF titers were immunoglobulin (Ig) M-negative with a reactive IgG of 1:256. Her physician prescribed 3 weeks of doxycycline for RMSF, and the fevers resolved. Subsequently, she was referred to an infectious disease specialist, who found repeat RMSF titers unchanged, which made acute RMSF unlikely. Additional testing identified a reactive *Brucella* SAT (1:640). When interviewed by NYSDOH staff, the patient reported that she was a laboratory worker (laboratory worker 2) at laboratory 2. Her initial blood culture specimen, which had originally been identified as *Micrococcus*, was reassessed by the commercial laboratory. The commercial laboratory referred the original isolate to the Wadsworth Center, where the isolate was identified as *B. melitensis*.

No evidence of exposure to *Brucella* spp., other than through occupational exposure, was identified for either laboratory worker. They denied traveling outside of the United States, consuming imported or domestic unpasteurized dairy products, knowing ill family or friends who may have traveled, attending events with potentially contaminated foods, or handling farm or laboratory animals. Both laboratory workers denied any accidental contamination or spills in the laboratory during the 6 months before their respective illnesses. Site visits to laboratories 1 and 2 were conducted by NYSDOH. On the open bench at laboratory 1, blood cultures were routinely subcultured onto agar. A syringe was used to directly plate contents of the blood culture media bottle onto agar. A Gram stain was then performed with additional contents from the syringe. The stain was fixed in an incubator. Subsequent biochemical tests, including a catalase test, which may generate aerosols by introducing hydrogen peroxide to the specimen, were performed on the open laboratory bench. At laboratory 2, subculturing occurred in a similar manner, but it took place in a class II biosafety cabinet. However, biochemical tests were performed on the open laboratory bench when Gram stain results indicated that spores were not present. Laboratory workers 1 and 2 wore gloves when processing specimens, and both denied having dermatitis or skin lesions on their hands.

After the diagnosis of brucellosis in these two laboratory workers, serum samples from their co-workers from laboratories 1 and 2 were tested by *Brucella* SAT; samples from seven of eight co-workers were nonreactive (<1:20). A co-worker of laboratory worker 1 had an initial agglutination titer of 1:40 (indeterminate) and 1 month later had a repeat titer of <1:20; she denied having symptoms.

To determine the source of these laboratory workers' infections, each laboratory reviewed Gram stains of blood culture specimens processed within 3 to 6 months of symptom onset. Laboratory worker 2 reviewed prior Gram stain slides from laboratory 2 and identified one slide containing a questionable coryneform bacillus. The Gram stain had originated from the blood culture of laboratory worker 1. This blood culture had been drawn from laboratory worker 1 during her visit to the emergency room of laboratory worker 2's hospital, early in November 2001. Approximately 2 months before her illness, laboratory worker 2 had personally processed laboratory worker 1's blood culture but had characterized the isolate as coryneform bacilli. Wadsworth Center staff reviewed the original Gram stain from laboratory worker 1's blood culture specimen and noted gram-variable organisms with similar size and shape to *Brucella* organisms. No isolate was available for confirmation. However, laboratory worker 2 did not identify any other Gram stains that resembled *Brucella* organisms during the period before her illness.

The likely source of laboratory worker 1's infection was found after reassessing Gram stains performed in laboratory 1 in August, September, and October 2001. A 76-year-old woman visited the emergency room served by laboratory 1 in early September 2001. The patient had had fever (temperature 38.3ºC) for 1 day and headache for 3 weeks. She received one dose of ciprofloxacin for a possible urinary tract infection and was discharged. After 4 days of incubation, a Gram stain of this patient's blood culture specimen showed tiny, gram-negative coccobacilli, reported as coryneform bacilli. As part of the investigation for the source of infection for laboratory worker 1, this patient's prepared Gram stain slide was referred to the Wadsworth Center, where small, gram-variable cocci were identified. More than 1 year after being seen in the emergency room, this patient was offered *Brucella* SAT and a repeat blood culture. She refused *Brucella* SAT, and her physician did not identify symptoms consistent with brucellosis. Six months after this identification, further experimental analysis by the Wadsworth Center with polymerase chain reaction tests performed on Gram stain material detected *B. melitensis* DNA (Wadsworth Center, NYSDOH, unpub. data). The patient was again contacted, and *Brucella* SAT (>1.5 years after her initial blood culture) showed a titer of 1:80 (indeterminate). Repeat blood cultures were negative. Upon interview, the patient denied any visits outside the United States since 1989, when she emigrated from Peru. She also denied consuming any unpasteurized products from the United States or abroad. An additional interview was scheduled, but the patient died unexpectedly.

Conclusions

Although brucellosis is a rare disease in the United States, its potential use as a bioweapon highlights the need for accurate and rapid identification (15). In this investigation, brucellosis was diagnosed weeks to years after initial

positive blood cultures were misidentified, and laboratory personnel were unknowingly exposed to the organism.

This investigation suggests that transmission occurred from the 76-year-old index patient to laboratory worker 1, on the basis of *B. melitensis* DNA found in the Gram stain material. Processing the index patient's blood culture specimen on an open laboratory bench was most likely the reason for laboratory worker 1's illness approximately 5 weeks later. The same mechanism of transmission probably occurred when laboratory worker 2 handled laboratory worker 1's blood culture specimen on the open bench for biochemical testing. In these instances, blood culture bottle media were transferred to slides and agar without much risk for aerosolization, since contents were manipulated with a syringe. Biochemical tests, however, included the catalase test, which creates bubbles as a result of exposure to 3% hydrogen peroxide in positive specimens. *Brucella* spp. are known to be catalase positive. Neither laboratory worker could identify any other possible sources of infection, and because brucellosis is a rare disease in New York, the connection between these three patients is plausible. With the initial interpretations of these Gram stains as gram-variable, which resulted in misidentification of the organism by three different laboratories, NYSDOH initiated an effort to educate clinical laboratories in New York State about the potential difficulties in characterizing the organism and the importance of primary Gram stain interpretation. Additional investigation into the staining properties of *Brucella* spp. under various conditions is now in progress and may help differentiate *Brucella* spp. from other organisms.

Because of immigration and foreign travel, brucellosis remains an occupational hazard for laboratory personnel, even in industrialized countries where animal control efforts have virtually eliminated the disease. Because of the nonspecific symptoms and the rarity of the disease in the United States, healthcare providers may not consider brucellosis in a differential diagnosis. However, eliciting travel and occupational histories may assist in diagnosis. Moreover, improved communication among healthcare providers and laboratory personnel should facilitate prompt and accurate identification and appropriate handling of the organism. Its potential use as a bioweapon necessitates that healthcare providers, as well as microbiologists in hospitals and commercial laboratories, be knowledgeable about the diagnosis, identification, and handling of *Brucella* spp.

Reporting brucellosis cases to public health officials is another component in protecting others from this disease. Public health officials should notify laboratory personnel who may have handled cultures taken from patients ultimately diagnosed with brucellosis. Early notification of exposed personnel could lead to their timely diagnosis and treatment, should symptoms occur, and could prevent further laboratory exposures.

At the time of this report, Dr. Noviello was an epidemic intelligence service officer at the Centers for Disease Control and Prevention working at the New York State Department of Health. Her projects included an evaluation of short-course latent tuberculosis infection treatment among inmates and investigations of foodborne outbreaks in New York State.

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Emerging Enteropathogenic *Escherichia coli* Strains?

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Escherichia coli strains of nonenteropathogenic serogroups carrying *eae* but lacking the enteropathogenic *E. coli* adherence factor plasmid and Shiga toxin DNA probe sequences were isolated from patients (children, adults, and AIDS patients) with and without diarrhea in Brazil. Although diverse in phenotype and genotype, some strains are potentially diarrheagenic.

Typical and atypical enteropathogenic *Escherichia coli* (EPEC) strains constitute two distinct groups of organisms that have in common the locus of enterocyte effacement (LEE), a pathogenicity island that promotes the development of attaching and effacing lesions (1,2). The LEE island encompasses the *eae* gene that encodes intimin, an outer membrane adhesin fundamental to the establishment of attaching and effacing lesions (1). Only typical EPEC strains bear the EPEC adherence factor (EAF) plasmid, in which a cryptic sequence used as a probe (EAF probe) to the category is located (1).

Various evidence suggests that atypical EPEC are closer to Shiga toxin–producing *E. coli* (STEC) (1), which cause diarrhea and hemolytic uremic syndrome (2). Although many STEC strains carry LEE, their main virulence mechanism is Shiga toxin(s) (Stx) production (2).

Twelve EPEC serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158) are recognized, but recent studies have shown that most typical EPEC strains fall into only certain O:H serotypes within these serogroups, which differ from those of atypical EPEC (1). Furthermore, *E. coli* strains of non-EPEC serogroups that carry *eae* but lack the EAF probe sequence and *stx* genes (*eae*+ EAF– *stx*– *E. coli*) have been detected (3–6), but their role in endemic diarrhea has not been established, and no precise understanding of them exists. Recently, we extensively characterized a collection of such strains from a single city in Brazil (6). To extend our knowledge on the diversity of *eae*+ EAF *stx*– *E. coli* strains of non-EPEC serogroups, we compared their occurrence in three distinct cities in Brazil and their genotypic and phenotypic characteristics.

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The Study

The strains we studied were collected from patients of low socioeconomic status in three cities: São Paulo and Ribeirão Preto, in São Paulo State, and Rio de Janeiro, in Rio de Janeiro State, Brazil. The São Paulo strains were collected from 505 diarrheic and 505 nondiarrheic children (1–4 years of age) who visited Hospital Infantil Menino Jesus (April 1989–March 1990) (7). These strains had been previously characterized for various traits (6); in the present study, we tested them for new gene sequences. The Rio de Janeiro strains were collected from 372 diarrheic and 74 nondiarrheic children ≤5 years of age at the Instituto de Puericultura e Pediatria Martagão Gesteira, a public hospital at the Federal University of Rio de Janeiro (January 1998–December 1999, and May–December 2001). Strains from Ribeirão Preto were derived from 294 diarrheic children (≤9 years of age) and adults (18–52 years), including 42 adults with AIDS. Fecal samples from these patients were sent to the Regional Laboratory of Instituto Adolfo Lutz by Hospital Santa Lydia and different clinics in the vicinity (August 2000–June 2002). This study has been approved by the Universidade Federal de São Paulo, Escola Paulista de Medicina Ethical Committee for human experimentation.

In all studies, five lactose-fermenting isolates and one nonlactose-fermenting isolate of each morphologic type, present in each fecal sample, were biochemically characterized as *E. coli*. Other well-established bacterial enteropathogens (*Salmonella* spp., *Shigella* spp., *Aeromonas* spp, *Campylobacter* spp., and *Yersinia enterocolitica*) and rotavirus were also searched for by standard methods (8).

All *E. coli* isolates were tested by colony hybridization with cloned or amplified genetic sequences for enterotoxigenic *E. coli*, enteroinvasive *E. coli*, EPEC (*eae* and EAF probes), STEC (*stx* probes), and enteroaggregative *E. coli*, as previously described (6). The *E. coli* strains that were *eae*+ EAF– *stx*– were serotyped at the Instituto Adolfo Lutz (National Reference Center for *E. coli* Serotyping) by using antisera O1 to O173 and H1 to H56.

In São Paulo and Rio de Janeiro, the *eae*+ EAF– *stx*– *E. coli* strains of non-EPEC serogroups occurred in similar frequencies in diarrheic and nondiarrheic children: 32 (6.3%) compared with 27 (5.3%), and 19 (5.1%) compared

with 4 (5.4%), respectively. In Ribeirão Preto, such strains were found in 17 (5.8%) patients: 13 from children (1 month–9 years of age) and 4 from adults with AIDS (27–52 years of age). A total of 99 strains (one from each patient) were selected for further analysis. These strains had diverse serotypes (Table 1); 25 (25.2%) strains were nonmotile, 3 were rough, and 47 (47.5%) did not react with the O antisera tested. Among the 49 O-typable strains, 29 serogroups and 35 serotypes were found. The most frequent serotype was O51:H40 (10.1%), which occurred in all three areas studied. Most of the other serotypes occurred in one or two strains.

All strains were tested for adherence to HeLa cells (3- and 6-hour assays) (9). Four of them promoted sporadic adherence, four were nonadherent, and one was cytodetaching. For 88 of the 90 adherent strains, the adherence patterns could only be determined in 6 hours. Seventy-two (80.0%) of the 90 strains had variations of the localized adherence (LA) pattern of typical EPEC, which is characterized by compact bacterial clusters (10). These variant patterns included the following: LA-like pattern, which showed loose bacterial clusters (11); a pattern that showed loose and compact clusters; and a pattern identical to LA, despite its detection in only 6 hours (LA6). Other less frequent patterns included the following: the diffuse adherence typical of diffusely adhering *E. coli*, the aggregative adherence typical of enteroaggregative *E. coli* (2), and an association of diffuse adherence and LA or of aggregative adherence and LA. These mixed patterns were retained when individual colonies were tested. The aggregative adherence/LA pattern (two strains) was only recognized in the 3-hour assays. The prevalence of the different patterns varied by area of study, but the variations of LA were the most prevalent in all (72.7%) (Figure 1).

The ability to promote attaching and effacing lesions was tested by the fluorescent actin staining test (FAS) (7) in 94 strains; the 5 nonadherent or cytodetaching strains were not tested. Seventy (74.4%) of the strains tested were positive: 43 (72.9%), 15 (65.2%), and 12 (70.2%) of the strains from São Paulo, Rio de Janeiro, and Ribeirão Preto, respectively. Moreover, four distinct segments of the LEE region were found in all strains studied, as detected by hybridization with specific LEE sequences (LEE A, B, C, and D) (12), which suggests that all bear a complete LEE region.

LEE insertion sites were detected by a combination of polymerase chain reaction (PCR) assays with primers for the *selC* junctions and for conserved sequences of *selC* and *pheU* (12,13). LEE was inserted in *selC* in 46 strains: 24 (40.7%), 13 (56.6%), and 9 (53.0%) strains from São Paulo, Rio de Janeiro, and Ribeirão Preto, respectively. In addition, LEE was probably inserted in *pheU* in 29 (49.1%) and 3 (13.0%) of the São Paulo and Rio de Janeiro strains, respectively. In 13 strains, LEE is probably inserted in another site, since both loci were intact. The LEE insertion site was undetermined in eight strains because both *selC* and *pheU* were disrupted, and the primers for the LEE junctions in *selC* yield no amplification. Strains with an undetermined LEE insertion site occurred in all three areas studied.

Table 1. Serotypes identified among *eae*+ EAF- *stx*- *Escherichia coli* strains outside the enteropathogenic *E. coli* serogroups[a]

Serotype (no. of strains)	Serotype (no. of strains)	Serotype (no. of strains)
O2ab:H45	O101:H33	ONT:H7 (3)
O2ab:HNT	O104:H-	ONT:H8 (4)
O4: H1	O104: H12	ONT: H9
O4: H16	O109:H9	ONT:H11
O11: H2	O115:H8	ONT:H19 (3)
O11: H16	O118:HNT (2)	ONT:H25
O13:H11	O121:H-	ONT:H29,31
O16:H-	O123:H19	ONT:H33 (3)
O19:H-	O124:H40	ONT:H34
O39:H-	O132:H8	ONT:H38
O41:H-	O145:H-	ONT:H40 (2)
O49:H10	O153:H7	ONT:H40,43 (2)
O51: H40 (10)	O154:H9	ONT:H46
O51: H-	O157:H16	ONT:HNT (3)
O63:H6 (2)	O162:H-	OR:H11,21,40
O66:H8	O162:H33	OR:H11,21
O70:H2	ONT:H- (16)	OR:H28
O85:H31 (3)	ONT:H2 (2)	
O98:H8	ONT:H6 (2)	

[a]NT, nontypable with antisera O1 to O173 and H1 to H 56; H-, nonmotile; R, rough strains.

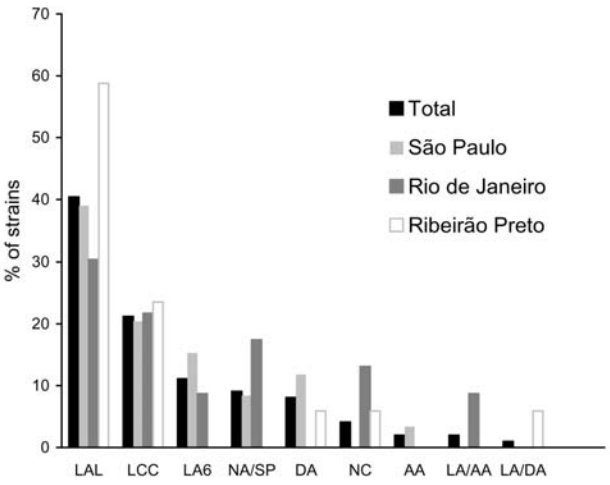

Figure 1. Prevalence of distinct adherence patterns in *eae*+ EAF- *stx*- *Escherichia coli* strains outside the enteropathogenic *E. coli* serogroups in three cities in Brazil. LAL, localized adherence-like; LCC, loose and compact clusters; LA6, localized adherence in 6-hour assay; NA/SP, nonadherent/sporadic; DA, diffuse adherence; NC, noncharacteristic; AA, aggregative adherence; LA/AA, localized and aggregative adherence; LA/DA, localized and diffuse adherence.

Strains were also tested for 24 DNA sequences of established or putative virulence properties of pathogenic *E. coli* by colony hybridization (6). DNA probes were obtained from cloned genes (*bfpA*, *perA*, *E-hly*, EAEC, *daaC*, *cdt*, *cnf*, *hly*) (6) or by PCR amplification, which used as templates the genomic DNA of EAEC prototype strains 042 (*aafC*, *aggR*, *aspU*, *shf*, *irp2*, *pet*, and *pic*) and 17-2 (*aggC* and *astA*); extraintestinal pathogenic strains (ExPEC) J96 (*pap*) and KS52 (*afa*), and *E. coli* HB101 (pANN 801-13) (carrying the *sfa* probe). PCR primers and assay conditions used were described previously (6,14).

Hybridization with 17 of the 24 sequences tested was detected among the strains; *hly* and *irp2* (31.3% each) and *astA* (29.3%) were the most frequent. Thirty-four different combinations of these 17 sequences were found (Table 2). Their prevalence varied by location, but 25 (73.5%) occurred in two or fewer strains. Among the less frequent combinations found, some were of genes of ExPEC and EAEC, and others of genes of EPEC (*bfpA)* and EHEC *(E-hly).* Moreover, 30.3% of the strains lacked all 24 DNA sequences tested, comprising the most frequent subgroup of strains in all three areas (Table 2). Although these strains carried only the *eae* gene and the four LEE probe sequences (LEE+ only strains), they may have carried virulence sequences other than those tested. Thus, one should not emphasize the virulence potential of these LEE+ strains solely on the basis of findings of significant differences in their frequencies between cases and controls.

DNA sequences similar to *bfpA* were detected in 14 (14.1%) of the 99 strains studied, however, only 2

Table 2. Prevalence of distinct combinations of virulence-related DNA sequences in *eae*+ EAF- *stx*- *Escherichia coli* strains outside the EPEC serogroups in three cities in Brazil[a]

	No. (%) of strains			
Genetic profile[b]	Total (n = 99)	São Paulo (n = 59)	Rio de Janeiro (n = 23)	Ribeirão Preto (n = 17)
eae	31 (31.1)	19 (32.2)	5 (21.8)	7 (41.1)
eae hly astA pet irp2	8 (8.1)	8 (13.6)	0	0
eae hly	6 (6.1)	5 (8.5)	0	1(5.9)
eae shf	5 (5.1)	1(1.7)	3 (13.1)	1(5.9)
eae irp2	5(5.1)	4 (6.8)	1(4.3)	0
eae perA bfpA astA	4 (4.0)	1(1.7)	3 (13.1)	0
eae perA bfpA	4 (4.0)	0	4 (17.4)	0
eae hly daaC afa astA pet irp2	3 (3.0)	3 (5.1)	0	0
eae perA	3 (3.0)	0	0	3 (17.6)
eae perA hly astA pet irp2	2 (2.0)	1(1.7)	0	1(5.9)
eae EHEC-*hly astA*	2 (2.0)	2 (3.4)	0	0
eae astA irp2	2 (2.0)	2(3.4)	0	0
eae bfpA	2 (2.0)	1(1.7)	1(4.3)	0
eae EHEC-*hly*	2 (2.0)	0	2 (8.7)	0
eae hly daaC afa pap sfa astA shf pet irp2	1(1.0)	1(1.7)	0	0
eae hly daaC afa shf irp2	1(1.0)	1(1.7)	0	0
eae perA bfpA hly pet	1 (1.0)	0	0	1(5.9)
eae perA hly daaC afa	1(1.0)	0	0	1(5.9)
eae perA bfpA astA irp2	1(1.0)	1(1.7)	0	0
eae hly pap afa irp2	1(1.0)	0	1(4.3)	0
eae hly daaC afa astA	1(1.0)	1(1.7)	0	0
eae hly astA shf irp2	1(1.0)	1(1.7)	0	0
eae perA bfpA hly	1(1.0)	0	0	1(5.9)
eae hly astA irp2	1(1.0)	1(1.7)	0	0
eae hly shf irp2	1(1.0)	1(1.7)	0	0
eae perA astA	1(1.0)	0	1(4.3)	0
eae EHEC-*hly bfpA*	1(1.0)	1(1.7)	0	0
eae hly shf	1(1.0)	1(1.7)	0	0
eae hly irp2	1(1.0)	1(1.7)	0	0
eae hly astA	1(1.0)	1(1.7)	0	0
eae astA shf	1(1.0)	0	0	1(5.9)
eae shf irp2	1(1.0)	0	1(4.3)	0
eae astA	1(1.0)	1(1.7)	0	0
eae cdt	1(1.0)	0	1(4.3)	0

[a]EPEC, enteropathogenic *Escherichia coli*; EHEC, enterohemorrhagic *E. coli.*
[b]All strains hybridized with the locus of enterocyte effacement (LEE) A, LEE B, LEE C, and LEE D probes constructed by McDaniel et al. (12), which suggested that they bear a complete LEE region.

expressed Bfp in Western blot experiments (not shown); these two strains also carried *perA* and presented AA/LA in 3 hours. The HeLa pattern of the remaining *bfpA*+ strains varied, but none of them had compact clusters in 3 hours, which is typical of LA. Thus, Bfp expression was found only in strains presenting aggregative adherence/LA in 3 hours, as in typical LA of EPEC (1).

PCR assays with specific primers for the variable region of intimin were used to identify five intimin types (α, β, γ, δ, and ε) (15,16). Most strains had a nontypable intimin (64.6%), but the distribution of these strains varied (approximately 70% in São Paulo and 29%–35% in Rio de Janeiro and Ribeirão Preto). Recently, new schemes were proposed to identify intimin subtypes, which were not tested (17,18). The prevalence of typable intimins varied among the three areas analyzed. Intimin subtypes β (11.1%) and γ (12.1%) prevailed, and intimin ε was not found (Figure 2). The intimin types of two strains were not determined because amplification products of the expected size were obtained with four intimin pairs of primers.

Conclusions

In this study, we sought to verify the frequency with which *eae*+ EAF– *stx*– *E. coli* strains of non-EPEC serogroups occur in persons of poor socioeconomic status in three Brazilian cities; we also compared these strains' genotypic and phenotypic characteristics. Although these strains occurred in 5% to 6% of the populations studied, including nondiarrheic children (in São Paulo and Ribeirão Preto), 73%–88 % of them were dissociated from other well-established enteropathogens (not shown).

Although O51:H40 was the most frequent serotype found and occurred in all three areas studied, the non-EPEC *eae*+ EAF– *stx*– strains comprised a large variety of serotypes, and many were O nontypable. Moreover, the strains had diverse adherence patterns and various combinations of pathogenic *E. coli* DNA virulence sequences; the prevalence of these properties varied among the areas studied. Altogether, these data show that *eae*+ EAF– *stx*– *E. coli* strains outside the EPEC serogroups are even more diverse than already observed (6). As we have emphasized previously, such diversity challenges the diagnosis of these putative pathogens (6).

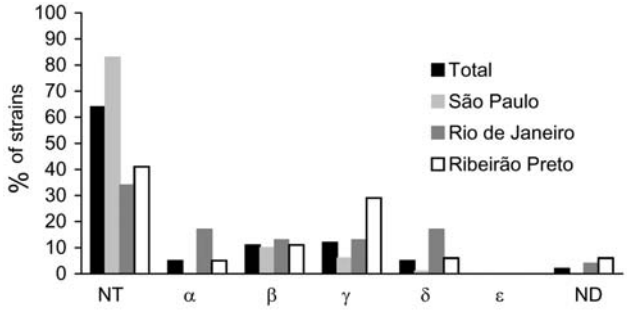

Figure 2. Intimin types in *eae*+ EAF– *stx*– *Escherichia coli* strains outside the enteropathogenic *E. coli* serogroups in three cities in Brazil. NT, nontypable with the sequences tested; ND, not done.

All strains carried an apparently complete LEE region, and approximately 75.0% of them had the potential to promote attaching and effacing lesions in HeLa cells, as detected by FAS. Thus at least these FAS+ strains are potentially enteropathogenic, since they are capable of inducing attaching and effacing lesions in vitro and may occur in diarrheic patients of various ages and in patients with AIDS. In the EPEC meeting held in 1995, a consensus definition of atypical EPEC was established, namely, that they are EAF–, *eae*+ strains that promote attaching and effacing lesions (19). Therefore, the FAS+ strains of our study could be classified as atypical EPEC. Whether these strains have additional virulence properties not present in typical EPEC remains to be elucidated. Studies on the virulence potential of selected strains at the cellular and molecular levels will certainly contribute to further understanding of this group of strains and aid in discriminating enteropathogenic strains within the group.

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Escherichia coli O157 Cluster Evaluation

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We investigated a multistate cluster of *Escherichia coli* O157:H7 isolates; pulsed-field gel electrophoresis subtyping, using a single enzyme, suggested an epidemiologic association. An investigation and additional subtyping, however, did not support the association. Confirming *E. coli* O157 clusters with two or more restriction endonucleases is necessary before public health resources are allocated to follow-up investigations.

Escherichia coli O157:H7 is an important cause of foodborne infections estimated to cause 73,000 illnesses and 60 deaths annually in the United States (1). Implementation of pulsed-field gel electrophoresis (PFGE) molecular subtyping has greatly improved *E. coli* O157:H7 surveillance and detection of outbreaks (2). PFGE subtyping was initially used to identify related isolates and support epidemiologic associations during outbreak investigations. Public health laboratories in the United States now routinely subtype all *E. coli* O157:H7 isolates by PFGE as part of a national molecular subtyping network (PulseNet) (2) after this practice proved instrumental in identifying outbreaks not detected by traditional epidemiologic methods (3). PulseNet laboratories initially digest isolates with a single enzyme and compare the resulting PFGE patterns by using commercial software (BioNumerics, St. Martens-Latem, Belgium) to determine whether patterns are shared by multiple isolates. These patterns are then communicated electronically to the Centers for Disease Control and Prevention (CDC) (Atlanta, GA), where PFGE patterns of isolates from different states are definitively compared. PulseNet policy states that isolates with potential epidemiologic significance that have indistinguishable patterns with a primary enzyme, should be digested with a secondary enzyme before extensive epidemiologic investigations are undertaken. Indistinguishable patterns should also be confirmed by submission to a central database (2). However, time constraints and the availability of sufficient resources prevent some laboratories from adhering to this policy.

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The Study

On July 5, 2000, the Michigan Department of Community Health's laboratory notified CDC of a cluster of five *E. coli* O157:H7 isolates, collected from May 25 to June 21, 2000, which shared an indistinguishable *Xba*I PFGE pattern. PulseNet staff confirmed that these isolates' patterns were indistinguishable and designated the pattern as PulseNet pattern EXHX01.0047. In 2000, this PFGE pattern represented approximately 2% of the *E. coli* O157 patterns in the PulseNet database. These Michigan isolates possessed genes only for Shiga toxin 2 (*stx*2) but not Shiga toxin 1 (*stx*1); approximately 30% of *E. coli* O157 isolates sent to CDC since 1983 expressed only *stx*2. From July through September 2000, six states (California, Michigan, New Jersey, New York, Ohio, and Texas) reported a total of 64 *E. coli* O157 isolates with PulseNet pattern EXHX01.0047, a value that exceeded expectation for this time of year and that prompted an epidemiologic investigation. Not all of these patterns were submitted to CDC's central database for confirmation. Fifty-one of these isolates were probed for Shiga toxin genes, and all possessed only *stx*2. Illness onsets ranged from April 1 through August 21, 2000, with a notable increase after late May 2000. The median age of case-patients was 13 years (range 1–91), and 38 (60%) were female; 36 (57%) of 64 were hospitalized, and hemolytic uremic syndrome developed in 9 (14%).

To determine the source of these *E. coli* O157:H7 infections, six state health departments (California, Michigan, New Jersey, New York, Ohio, and Texas) and CDC initiated an epidemiologic investigation. Informed consent was obtained from all patients or their parents or guardians and human experimentation guidelines of the U.S. Department of Health and Human Services were followed. These data were collected as part of an outbreak investigation and therefore were exempt from formal institutional review board aprovals.

Through hypothesis-generating interviews with 19 infected persons, 11 food exposures were reported by ≥50% of interviewees or were reported in substantial excess relative to that food's frequency of consumption in the general population (4). In a case-control study that used a survey instrument that focused on these 11 food exposures, controls were matched to case-patients by sex and age group, and were asked about exposures during the

same 5-day period before the matching case-patient's illness onset. Controls were contacted and identified by using sequential-digit dialing beginning with the matching patient's telephone number.

Twenty-eight case-patients and 69 matched controls were enrolled (2.46 controls per patient). The median age was 13.5 years and 50% were female; case-patients did not differ significantly from controls in terms of sex or age. In matched univariate analysis by using logistic regression with stratification (LogXact version 2.1.1, Cytel Software Corporation, Cambridge, MA), only broccoli was significantly associated with illness (matched odds ratio [mOR] 3.65, p = 0.04); 14 (58%) of 24 case-patients, and 19 (31%) of 62 controls reported eating broccoli. Although none of the three foods that contained ground beef were individually associated with illness, consumption of "any hamburger" (a composite variable) was significant (mOR 7.30, p = 0.01), reported by 20 (87%) of 23 patients and 28 (55%) of 51 controls. In multivariate analysis, only eating "any hamburger" remained significantly associated with illness (OR = 6.13, p = 0.02).

Ground beef eaten by case-patients was recovered from three households; samples from two households (in New Jersey and California) yielded *E. coli* O157:H7 isolates that were indistinguishable from PulseNet pattern EXHX01.0047. One of these isolates was tested for Shiga toxin and produced only *stx*2. Using information from these two cases and additional information regarding likely ground beef sources for the original Michigan cases, the U.S. Department of Agriculture performed a traceback; however, an extensive investigation did not identify any common supplier for the two samples of ground beef.

We performed a retrospective review of available isolate patterns received by the PulseNet national database after the case-control study and traceback had been completed. Four additional states (Florida, Indiana, Massachusetts, and Washington) had reported *E. coli* O157 isolates with *Xba*I patterns that were indistinguishable from PulseNet pattern EXHX01.0047. Among the 46 submitted *Xba*I patterns from states reporting a possible match to PulseNet pattern EXHX01.0047, analysis at CDC indicated that 38 were indistinguishable and that 6 differed by one band from the PulseNet pattern EXHX01.0047 (Figure 1). Furthermore, among the 38 isolates confirmed as PulseNet pattern EXHX01.0047, digestion of 13 isolates with the restriction enzyme *Bln*I produced PFGE patterns that sorted into multiple distinct clusters (Figure 2).

Conclusions

Identifying outbreaks of *E. coli* O157:H7 infections by routinely subtyping isolates using PFGE is a relatively new phenomenon (2,3). Traditionally, PFGE has been used to support or refute the likelihood of epidemiologic relatedness among case-patients and suspect food vehicles in epidemiologic investigations. In this instance, the converse occurred; the results of routine PFGE subtyping (*Xba*I) of *E. coli* O157:H7 isolates prompted a large, multistate epidemiologic investigation. Isolates were potentially related because 1) the PFGE patterns obtained with one restriction enzyme (*Xba*I) were reported to be indistinguishable and a relatively uncommon pattern, and 2) the isolates shared a Shiga toxin profile that was relatively uncommon among *E. coli* O157 (*stx*2 only). A rigorous case-control study implicated a widely consumed food vehicle responsible for multiple past outbreaks of *E. coli* O157 infections: ground beef (5). This study and the isolation of two *E. coli* O157 with matching PFGE patterns from ground beef consumed by case-patients prompted two extensive traceback investigations. However, no common source could be identified. Subsequent digestion of patient isolates with a second enzyme showed that they were actually part of multiple, small clusters and that the illnesses were thus unlikely to be related to a common source.

Investigation of suspected multistate outbreaks requires substantial public health resources (6). This investigation involved more than 50 federal, state, and local staff. *E. coli* O157:H7 infections can cause serious and potentially life-threatening illness that may also engender legal action. Public health authorities must ensure that linkage of illnesses to an outbreak be as complete and accurate as possible. Rapid identification of the infections' source can avert many potential illnesses. Earlier studies demonstrated the value of subtyping *E. coli* O157:H7 isolates with two or more restriction endonuclease digestions or using other subtyping methods, such as phage typing, to determine whether such isolates are truly related, even if these isolates have produced matching patterns using a single enzyme digestion (7–9). More recently, in the absence of epidemiologic data, single enzyme PFGE has been found to be a poor measure of genetic relatedness (10). Since 1998, the PulseNet Task Force has recommended the use of at least two enzyme digestions for optimal subtyping of *E. coli* O157 isolates. However, because of resource limitations, many state and local public health laboratories initially subtype *E. coli* O157 isolates with *Xba*I enzyme and perform subtyping with a second enzyme only if clusters are identified and personnel and resources are in place to do so. This

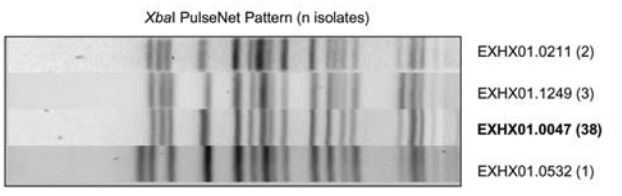

Figure 1. *Xba*I-generated pulsed-field gel electrophoresis patterns for *Escherichia coli* O157 isolates reported as indistinguishable from PulseNet pattern EXHX01.0047.

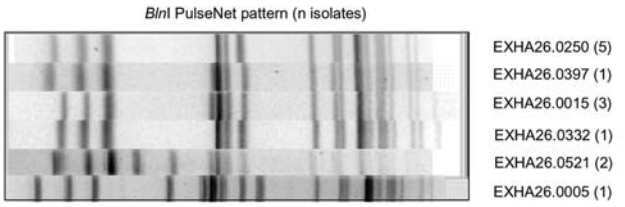

Figure 2. *Bln*I-generated pulsed-field gel electrophoresis patterns for *Escherichia coli* O157 isolates that generated patterns indistinguishable from PulseNet pattern EXHX01.0047 by using *Xba*I.

investigation lends further support to the conclusion that when clusters of *E. coli* O157 are detected on the basis of subtyping data only (i.e., in the absence of any epidemiologic data), digestion with two or more endonucleases is warranted, even if the isolates appear to share a primary enzyme pattern or possess other microbiologic evidence of clonality (e.g., Shiga toxin profile). Furthermore, these findings underscore the importance of having a centralized database team that can rapidly verify reports of clusters from participating PulseNet laboratories and assist in determining whether isolates are likely to be part of an outbreak and whether a rapid, large-scale epidemiologic investigation and traceback would be warranted.

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Mycobacterium triplex Pulmonary Disease in Immunocompetent Host

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Mycobacterium triplex, a recently described, potentially pathogenic species, caused disease primarily in immunocompromised patients. We report a case of pulmonary infection due to this mycobacterium in an immunocompetent patient and review the characteristics of two other cases. In our experience, *Mycobacterium triplex* pulmonary infection is unresponsive to antimycobacterial chemotherapy.

Nontuberculous mycobacteria (NTM) are ubiquitous organisms, commonly isolated from environmental and animal sources (1), whose pathogenicity may vary according to the host's immune status. Although exposure to NTM frequently causes no symptoms, clinical manifestations may range from hypersensitivity reactions (2) to destructive, even fatal, lung disease. In the case of lung disease caused by NTM, clinical and radiologic features are sometimes indistinguishable from those seen in lung disease caused by *Mycobacterium tuberculosis* complex (MTB). The clinical importance of NTM is often difficult to determine, especially in patients with chronic, preexisting lung disease; criteria for diagnosing disease caused by NTM, most recently updated by the American Thoracic Society in 1997 (3), need to be properly fulfilled.

M. triplex was first described in 1996 (4). Investigators reported a group of slowly growing, nonpigmented mycobacteria, resembling *M. simiae* or *M. avium* complex (MAC) in biochemical tests, which did not react with the commercial probe designed for MAC. The primary characterization of this new organism relied on conventional biochemical tests and analysis of mycolic acids with high-performance liquid chromatography (HPLC), but conclusive evidence was based on sequencing the 16S rRNA hypervariable region. In HPLC analysis, *M. triplex* produces a triple-clustered mycolic acid profile closely related to those of *M. simiae*, *M. genavense*, and the recently described *M. sherrisii* (5), but practically indistinguishable from that of *M. lentiflavum*, a novel mycobacterium characterized by Springer et al. (6). Phylogenetic studies showed that *M. triplex* and *M. lentiflavum* are closely related to *M. simiae* and *M. genavense*.

M. triplex has been reported to cause episodic infection in AIDS patients or in those with other immunocompromising diseases (7–9). We present the case of an apparently immunocompetent patient with pulmonary disease caused by this mycobacterium and review the characteristics of two similar cases.

The Case

On January 2002, a 54-year-old, HIV-negative, white woman was referred to the outpatient pulmonary service because of persistent cough lasting >2 years and fatigue. She lived in an urban area, had smoked cigarettes (40 packs/year) for several years, but did not report any history of alcoholism or use of immunosuppressive drugs. Results of physical examination and routine laboratory tests, including a standard tuberculin skin test, were unremarkable. A chest x-ray showed considerable fibrotic interstitial changes associated with patchy parenchymal shadowing in both lower fields and multiple thin-walled cavitary lesions (0.7–3.5 cm) in both upper lobes. A computed tomographic (CT) scan of the chest confirmed the above lesions, including multifocal bronchiectases in the right middle lobe and multiple small nodules in the lower lobes. Smears for acid-fast bacilli (AFB) were positive on two bronchial washing samples. These specimens produced negative results when tested by a commercial strand displacement amplification assay (Becton Dickinson Biosciences, Sparks, MD) specific for MTB. Chemotherapy with isoniazid, rifampin, and ethambutol was started. Cultures produced a slow-growing, nonpigmented mycobacterium that was identified in June 2002 as *M. lentiflavum*, according to the HPLC pattern of mycolic acids. One more bronchial specimen collected in mid-June was AFB smear-positive and yielded the same organism as was detected in January. Isolates were considered clinically relevant, and chemotherapy was changed to ethambutol and clarithromycin.

In December 2002, after 6 months of treatment, a CT scan did not show reduction of pulmonary lesions, although the general condition of the patient had slightly improved. Smears from one out of four additional bronchial washing samples were positive for AFB, and all specimens yielded the same mycobacterium previously detected. A more accurate evaluation of clinical isolates showed that their phenotypic pattern was different from that of *M. lentiflavum* by results of some biochemical tests and the absence of pigmentation. Further gene sequencing study of the 16S rDNA showed that our isolates exhibited

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100% homology with the reference strain of *M. triplex* (ATCC 70071). In March 2003, although chemotherapy was well tolerated, microbiologic tests of two bronchoalveolar lavage (BAL) specimens continued to be AFB smear- and culture-positive. Consequently, chemotherapy was changed to include levofloxacin, ethambutol, and clarithromycin. In October 2003, chest x-ray examination and a CT scan showed a slight reduction in the size of lung cavities. The patient continues to receive medication, and the radiologic picture has not improved.

Microbiologic Aspects

After standard N-acetyl-L-cysteine-sodium hydroxide decontamination (10), bronchoaspirate washing and BAL specimens were stained with routine Ziehl-Neelsen and cultured by a combination of the radiometric Bactec system (Becton Dickinson Biosciences) and Löwenstein-Jensen medium. Recovered strains were tested with a commercial multiplex line probe assay (Inno-LiPA Mycobacteria v2, Innogenetics, Ghent, Belgium) specific for MAC and 16 other different mycobacterial species (11). Further identification studies were performed by mycolic acid HPLC analysis (12) and 16S rDNA sequencing (13). Drug susceptibility pattern was determined in liquid medium by using the radiometric macrodilution method developed for MAC (14).

M. triplex strains were repeatedly isolated on both liquid and solid media (Table 1). An extended panel of biochemical and cultural tests was used for conventional identification (10) (Table 2). The hybridization test performed with the multiplex line probe assay was negative apart from the genus-specific line probe. Final identification was achieved by 16S rDNA gene sequencing. In fact, while HPLC profile was poorly discriminative between *M. lentiflavum* and *M. triplex* (Figure 1), 16S rDNA sequencing showed 100% homology with the *M. triplex* reference strain, which allowed the attribution of all our strains to this species (Figure 2). MICs (μg/mL) performed on the first isolate were the following: streptomycin 6.0, isoniazid >0.5, rifampin >2.0, ethambutol 7.5, ciprofloxacin 4.0, clarithromycin 8.0, amikacin 8.0, ethionamide 2.5, and rifabutin 0.5. Drug susceptibility testing was completed after 6 days. Tentative interpretations of MIC results, according to NCCLS M24-A (17) and data from Heifets (18), are reported in Table 1.

Conclusions

A search of the literature from 1996 (when *M. triplex* was first described) to January 2004 yielded two reports of pulmonary infections in immunocompetent patients (15,16). In those reports, clinical and radiographic features of *M. triplex* pulmonary infection did not differ substantially from those of TB and other NTM. Cough, hemoptysis, and fatigue were the primary symptoms, while radiographic studies found pulmonary nodules most commonly, followed by lung infiltrates, multifocal bronchiectasis, and cavitations (Table 1). No patient had underlying diseases when pulmonary infection with *M. triplex* was diagnosed, and tuberculin skin test results were negative or not reported. Although a history of preexisting pulmonary lesions could not be documented for these patients, repeated isolation of mycobacteria from different respiratory samples and the absence of other possible causes of pulmonary disease suggest that *M. triplex* was likely to cause symptomatic infection rather than colonization (3).

M. triplex strains were isolated on both liquid and solid media in our case, while culture media were not reported in the other two previously published cases. One case

Table 1. Clinical and microbiological features of pulmonary infection with *Mycobacterium triplex*[a]

Characteristic	Patient described in reference 15	Patient described in reference 16	Our patient
Age/Sex	67/F	54/F	54/F
Symptoms	Hemoptysis	Cough, hemoptysis, fever, fatigue	Cough, fatigue
Findings	Bronchiectases, lung nodules	Lung infiltrates and nodule (0.3 cm)	Bronchiectases, lung nodules, cavitations
Collected samples (no.)	Bronchial aspirate (1), sputum (3)	BAL (2), sputum (4)	Bronchial aspirate (7), BAL (2)
Smear-positive	None	None	6
Culture-positive	3	3	9
Mean no. CFU/mL (range)	NR	NR	693 (144–2,772)
In vitro testing			
S	RMP, SM, CLA	NR	CLA
I	CIP	NR	AN, CIP, EMB, ETH, RBT, SM
R	EMB, INH, PZA	NR	INH, RMP
Therapeutic schedule (mo.)	RMP, CIP, EMB, CLA (18)	RMP, INH, CLA (NR)	INH, RMP, EMB (6); EMB, CLA (9); LVX, CLA, EMB (9)
Outcome	Healed	NR	Slight improvement

[a]BAL, bronchoalveolar lavage; NR, not reported; S, susceptible; I, moderately susceptible; R, resistant; RMP, rifampin; SM, streptomycin; CLA, clarithromycin; CIP, ciprofloxacin; AN, amikacin; EMB, ethambutol; ETH, ethionamide; RBT, rifabutin; INH, isoniazid; PZA, pyrazinamide; LVX, levofloxacin.

Table 2. Biochemical characteristics of the described isolate compared to those of *Mycobacterium lentiflavum* and *M. triplex*

Characteristic	Our isolate	*M. lentiflavum* (6)	*M. triplex* (4)
Niacin	–	–	–
Nitrate reduction	+	–	+
Thermostable catalase	+	+	+
Tween 80 hydrolysis (10 days)	–	–	–
Tellurite reduction	–	–	NR
Arylsulfatase (3 days)	–	–	–
Urease	+	–	+
Catalase >45 mm	–	–	+
Photochromogenicity	–	–	–
Scotochromogenicity	–	+	–
Growth at 30°C	+	+	+
Growth at 37°C	+	+	+
Growth at 45°C	–	–	–
McConkey w/o CV	–	–	–
Tolerance to NaCl (5%)	–	–	–
Tolerance to TCH (5 mg/mL)	+	+	+
Growth rate	Slow	Slow	Slow
Colonial morphology	Smooth	Smooth	Smooth

[a]–, negative; +, positive; NR, not reported; CV, crystal violet; TCH, thiophene-2-carboxylic acid hydrazide.

reported an extended panel of biochemical and cultural tests for conventional identification that showed the absence of pigment and positive reactions to nitrate reductase, urease, and semiquantitative catalase as the most useful characteristics for tentative identification (15). All strains failed to hybridize with the commercially available genetic probe for MAC (Accuprobe, Gen-Probe Inc., San Diego, CA), so all reported cases could be definitively identified by 16S rRNA gene sequencing and similarity search with the BLAST alignment software (www.blast.genome.ad.jp). One strain showed 100% homology with the reference strain, while another showed homology of 99.5% and, therefore, despite its close relationship, was regarded as a variant of *M. triplex* (15).

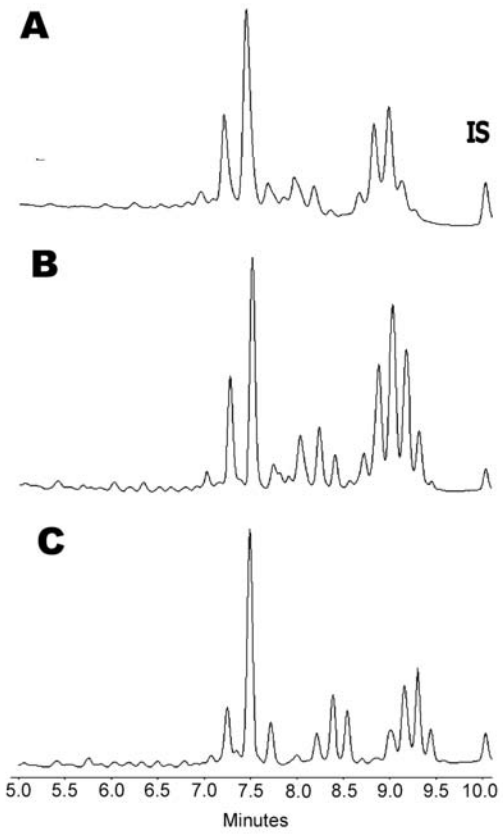

Figure 1. Comparison of high-performance liquid chromatography phenotypes of A) *Mycobacterium triplex*, B) *M. lentiflavum*, and C) *M. simiae*. IS; internal standard.

Patients (including our case-patient) were given different treatment regimens with two to four antimicrobial agents. Ethambutol, rifampin, clarithromycin, and ciprofloxacin were mainly used (Table 1). Clinical improvement, as defined by resolution of symptoms and radiographic findings (infiltrates and cavitary lesions), was obtained within 10 months after therapy was initiated in one of three patients. One patient improved when clarithromycin and ciprofloxacin were added to the regimen, while another was reported to have improved after drug therapy was initiated (16). Our patient was still sputum smear and culture–positive 2 years after therapy was initiated, despite exhibiting minor evidence of clinical and radiologic improvement.

Our findings show that *M. triplex* infrequently causes pulmonary disease in immunocompetent persons. Treatment with a three- or four-drug combination, including clarithromycin, ciprofloxacin, and ethambutol, was shown to be associated with reduced symptoms and good clinical outcome. Although at present only 16S rDNA sequencing can identify *M. triplex*, presumptive identification can be made when a slow-growing, nonpigmented NTM reduces nitrates, produces urease and semiquantitative catalase, and exhibits a three-clustered HPLC profile.

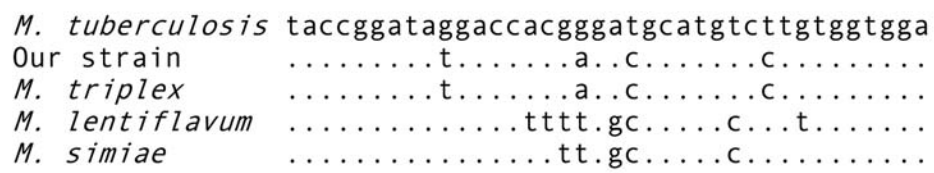

Figure 2. Sequence alignment of the hypervariable region A within the 16s RNA gene of the studied isolate and related species. *Mycobacterium tuberculosis* was used as the reference sequence. Nucleotides different from those of *M. tuberculosis* are indicated; dots indicate identity.

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Arcobacter Species in Humans[1]

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During an 8-year study period, *Arcobacter butzleri* was the fourth most common *Campylobacter*-like organism isolated from 67,599 stool specimens. Our observations suggest that *A. butzleri* displays microbiologic and clinical features similar to those of *Campylobacter jejuni*; however, *A. butzleri* is more frequently associated with a persistent, watery diarrhea.

Campylobacter is the most common cause of acute bacterial enteritis in the United States and many other industrialized countries (1,2). When the diagnosis of infection is based exclusively on culturing on selective media, >95% of *Campylobacter* infections are caused by *Campylobacter jejuni* or *C. coli*. However, with refinements in isolation and identification methods, other related species such as *C. upsaliensis*, *C. jejuni* subsp. *doylei*, *C. fetus* subsp *fetus*, *C. concisus*, *Arcobacter butzleri*, *Helicobacter fennelliae*, and *H. cinaedi*, have emerged as potential enteric human pathogens (3). Since most laboratories do not use appropriate culture conditions to detect all *Campylobacter* spp. and related organisms or do not identify isolates to species level, data on the incidence and clinical importance of these non–*C. jejuni/coli* organisms are scarce.

During the past decade, improvements in isolation techniques in veterinary medicine have led to the discovery of *Arcobacter* spp. as animal pathogens. Members of the genus *Arcobacter* are aerotolerant *Campylobacter*-like organisms. They were first isolated from aborted bovine fetuses by Ellis in 1977 (4). *Arcobacter* spp. differ from *Campylobacter* spp. by their ability to grow at lower temperatures and in air (5). Later, *Arcobacter* infections in humans were also described. Two species, *A. butzleri* and, more rarely, *A. cryaerophilus,* have been associated with enteritis and occasionally bacteremia (6–9). Patients with *A. butzleri* infections report diarrhea associated with abdominal pain; nausea and vomiting or fever also occur (10,11). A third species, *A. skirrowii*, has recently been isolated from a person with chronic diarrhea (12). Despite these occasional reports, the contribution of *Arcobacter* species to human diarrhea is still unknown. The aim of our study was to compare the prevalence and the clinical features of *A. butzleri* isolated from stools with those of *C. jejuni*.

The Study

From January 1995 to December 2002, all stool samples submitted to two hospital laboratories serving the Brugmann, Queen Fabiola, and Saint-Pierre University Hospitals in Brussels, Belgium, were examined macroscopically for consistency, gross blood, and mucus and microscopically for parasites, leukocytes, and erythrocytes. These samples were also cultured for common bacterial pathogens. Stool samples of patients <2 years of age were also evaluated for rotavirus and enteric adenovirus since viral diarrhea is mainly seen in young children.

A specific culture protocol for the recovery of *Campylobacter* spp. and related organisms consisting of one solid selective medium and a filtration method was systematically applied. In the selective agar method, the fecal suspension (approximately 1 g/mL of saline) was plated onto Butzler's medium comprising Mueller-Hinton agar (Oxoid Ltd, Basingstoke, United Kingdom) containing 5% sheep blood and the antimicrobial supplement cefoperazone 30 mg/L, rifampicin 10 mg/L, and amphotericin B 2 mg/L (Institute Virion, Rüschlikon, Switzerland) (13). The plates were incubated for 48 h at 42°C in a microaerobic atmosphere (5% O_2, 6% H_2, 10% CO_2, and 79% N_2). The plates were examined daily for growth of *Campylobacter* species.

The membrane filtration method was performed according to Lopez (14). Stool samples were diluted 1:5 in Brucella broth. Cellulose acetate filters (50 mm in diameter) with a pore size of 0.45 μm were placed on the surface of Mueller-Hinton agar plates containing 5% sheep blood. Eight drops of the fecal suspension were placed on the top of the membrane and allowed to filter passively for 30 min at 37°C in air. After filtration, the filters were removed, and the plates were incubated at 37°C in a microaerobic atmosphere for up to 10 days.

Two specific procedures for isolating *Arcobacter* were used successively. Until April 1995, we used a membrane filtration technique (15). Subsequently, we switched to

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[1]Presented in part at the 11th International Congress on Infectious Diseases, Cancun, Mexico, Mar 4–8, 2004. (Abstract P09.030); and at the 12th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Aarhus, Denmark, Sept 6–10, 2003 (Abstract K-07).

direct plating on *Arcobacter* selective medium (16), for which 0.5 g of stool samples was injected into an enrichment broth (Brucella broth + antimicrobial supplement consisting of piperacillin 75 mg/L, cefoperazone 25 mg/L, amphotericin B 10 mg/L, trimethoprim 20 mg/L, and cycloheximide 100 mg/L + 5% laked horse blood) and incubated for 24 h at 25°C in a microaerobic atmosphere. After incubation, 40 µL of enriched broth was plated onto *Arcobacter* selective medium (16). The plates were then incubated for 3 days at 25°C in a microaerobic atmosphere and examined daily.

Gram-negative, motile, spiral, or curved rods were identified as *Campylobacter* by morphologic and biochemical characteristics consistent with the genus *Campylobacter*, as recommended by Vandamme et al. (5). A complementary distinction between *A. butzleri* and *A. cryaerophilus* was achieved by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis of whole-cell proteins and by a multiplex polymerase chain reaction assay (17).

To assess the pattern of clinical disease, patients with *A. butzleri* infection were matched against three randomly selected control patients with *C. jejuni* infection. To achieve this, all charts of patients with *A. butzleri* infection and controls were reviewed retrospectively by combining the records of the medical and microbiology departments. A structured, close-ended questionnaire was used to collect the patient's history, age, sex, status (outpatient or hospitalized for >48 h) and history of international travel. The clinical history included diarrhea within the preceding 3 months, duration of symptoms, nature of the diarrhea (watery or bloody and presence of cells), intensity of fever, nausea or vomiting, abdominal pains, asymptomatic carriage (routine screening of enteric pathogens in stools of HIV-infected persons, other immunocompromised states, and patients from a foreign country referred for surgery), and underlying disease. Diarrhea was defined as at least three unformed or liquid stools per day for at least 3 days. Because of the study's retrospective nature, we could only classify diarrhea as acute (duration <15 days) or chronic (duration >15 days). Treatment history included antimicrobial history and use of intravenous fluid therapy, and, finally, clinical status after 1 month of treatment (cure or persistent diarrhea).

All parameters were compared between index patients and matched controls. Comparisons were made by Pearson χ^2 test for 1:3 control data. Odds ratios (OR) and 95% confidence intervals (CI) were calculated.

From January 1995 to December 2002, a total of 67,599 stool specimens from 40,995 patients were submitted for bacteriologic stool culture. These cultures yielded *Campylobacter* and related organisms in 1,906 patients, *Salmonella* species in 1,720 patients, and *Shigella* species in 244 patients. Other identified pathogens include enteropathogenic *Escherichia coli* (137 patients), *Yersinia enterocolitica* (87 patients), *Plesiomonas* spp. (22 patients), and *Aeromonas* spp. (21 patients).

Among the 1,906 *Campylobacter* and related organisms isolated during the study period, 77.2% were *C. jejuni*, 11.4% were *C. coli*, and 4.5% were *C. upsaliensis* (Table 1). Ninety-seven *Arcobacter* isolates were obtained from 77 patients. Among them, *A. butzleri* was the most frequently isolated species (84 isolates from 67 patients), accounting for 3.5 % of the *Campylobacter* and related organisms bacterial group. Thirteen *A. cryaerophilus* isolates from 10 patients were obtained, but no *A. skirrowii* isolates were found. Other species, such as *C. concisus, C. fetus*, *C. curvus*, *C. lari*, and *C. hyointestinalis*, were also found in small numbers (Table 1).

Table 1. Distribution of *Campylobacter* spp. and related organisms isolated from 1,906 patients,[a] January 1995–December 2002, and comparison of recovery by isolation method used[b]

Species	No. (%)	No. of patients positive for *Campylobacter* by one medium or a combination of media						
		BSM	ASM	FM	BSM + FM	BM + ASM	ASM + FM	All methods
Campylobacter jejuni subsp. *jejuni*	1,471 (77.2)	1,353	12	1,076	1,471	1,353	1,081	1,471
C. coli	218 (11.4)	199	5	174	218	199	174	218
C. upsaliensis	85 (4.5)	7	0	85	85	7	85	85
Arcobacter butzleri	67 (3.5)[c]	3	65	5	5	65	65	67
C. concisus	27 (1.4)	0	0	27	27	0	27	27
C. fetus subsp. *fetus*	11 (0.6)	2	0	9	11	2	9	11
A. cryaerophilus	10 (0.5)	0	9	1	1	9	10	10
C. curvus	9 (0.5)	0	0	9	9	0	9	9
C. lari l	3 (0.2)	1	0	3	3	1	3	3
C. hyointestinalis	2 (0.1)	0	0	2	2	0	2	2
Helicobacter pullorum	2 (0.1)	0	0	2	2	0	2	2
C. sputorum	1 (0.1)	0	0	1	1	0	1	1
Total	1,906 (100.0)	1,565	91	1,394	1,835	1,636	1,468	1,906

[a]At the Saint-Pierre, Brugmann, and Queen Fabiola University Hospitals.
[b]BSM, Butzler selective medium; ASM, *Arcobacter* selective medium; FM, filtration method.
[c]Two of the 67 *A. butzleri* isolated were recovered by the filtration method (15) in use up to April 1995.

Medical records were available for 61 of the 67 patients with *A. butzleri* infection. Ages of patients with *A. butzleri* infection were 30 days–90 years; there were slightly more female than male patients. Fourteen patients were hospitalized for >48 h. Four patients had traveled abroad before onset of symptoms. Ten patients had underlying disease: 4 were HIV seropositive, and 3 were immunocompromised (postrenal graft, celiac disease, and chemotherapy for cerebellar astrocytoma). Other chronic illnesses included dementia (n = 1), insulin-dependent diabetes mellitus (n = 1), and hepatitis C (n = 1).

Thirty-one patients complained of acute diarrhea (≥24 watery stools) lasting for 3 to 15 days, and 10 had persistent or recurrent diarrhea lasting >2 weeks–2 months. Six patients had abdominal pain without diarrhea. Twelve patients were asymptomatic. Sixteen patients received antimicrobial therapy, but only 7 were treated empirically with an antimicrobial agent for which the strain was susceptible. The symptoms resolved in all patients except one, regardless of the antimicrobial agent used. Among patients treated symptomatically, three patients had persistent or recurrent symptoms.

Sixty-seven patients with *A. butzleri* infection were matched against 201 patients with *C. jejuni* infection. The age and sex distributions were similar for patients colonized by each species. No significant differences in international travel were observed. However, proportionally fewer patients with *A. butzleri* (79.1%) were treated as outpatients than those with *C. jejuni* (90.5%) (OR 0.40, 95% CI 0.17–0.90) (Table 2).

Rectal bleeding, inflammatory exudates, or both were significantly less common in *A. butzleri* than in *C. jejuni* infection (OR 0.15, 95% CI 0.05–0.46). A concomitant infection with another enteric pathogen occurred in a higher proportion of patients with *A. butzleri* infection than patients with *C. jejuni* infection, but the difference was not significant. Twelve patients with *A. butzleri* infection had a coinfection with one of the following enteric pathogens: *Salmonella enterica* (n = 4) (2 ser. Enteritidis, 1 ser. Typhimurium and 1 ser. Virchow), *Rotavirus* (n = 3), *C. jejuni* (n = 2), *Giardia lamblia* (n = 2), and *Clostridium difficile* toxin B positive (n = 1).

In 24 patients with *Campylobacter jejuni* infection, we found a coinfection with one another enteric pathogen: *S. enterica* (7) (3 ser. Enteritidis, 3 ser. Typhimurium, and 1 ser.Virchow), *Rotavirus* (5), *Adenovirus* (4), *Giardia lamblia* (2), *Shigella flexneri* (2), *Yersinia enterocolitica* (1), and *Clostridium difficile* toxin B positive (1). In two additional cases of campylobacteriosis, we found a coinfection with two other enteric organisms: one patient was infected with *S. dysenteriae* and *Hymenolepis nana* and the other patient with *Rotavirus* and *Salmonella enterica* serotype Typhimurium.

To assess the prevalence of *A. butzleri* and *Campylobacter jejuni* in diarrheic stool specimens, we considered only stools with loose or liquid macroscopic aspect as diarrhea. Among the 67,599 stool specimens received, 12,413 were solid stools, and 55,186 were diarrheic. Among the diarrheic stool specimens, we isolated *A. butzleri* and *C. jejuni* in 77 (0.14%) and 3,209 (5.81%)

Table 2. Case-control study of microbiologic and clinical features of patients with *Arcobacter butzleri* and *Campylobacter jejuni* infection

Features/treatment/outcome	No. patients with *A. butzleri* infection (%)	No. patients with *C. jejuni* infection (%)	OR (95% CI)
Microbiologic features			
Erythrocytes in stool	4/67 (6.0)	59/201 (29.4)	0.15 (0.05–0.46)
Leukocytes in stool	2/67 (3.0)	36/201 (17.9)	0.14 (0.02–0.62)
Associated organisms	12/67 (17.9)	24/201 (11.9)	1.61 (0.71–3.63)
Clinical features			
Diarrhea	41/61 (67.2)	149/191 (78.0)	0.58 (0.29–1.14)
Acute diarrhea	31/61 (50.8)	140/191 (73.3)	0.38 (0.20–0.79)
Persistent diarrhea	10/61 (16.4)	9/191 (4.7)	3.97 (1.40–11.33)
Watery diarrhea	31/61 (50.8)	61/191 (31.9)	2.20 (1.18–4.13)
Fever, temperature >38.5°C	20/61 (32.8)	83/191 (43.5)	0.63 (0.33–1.21)
Nausea, vomiting, or both	17/61 (27.9)	47/191 (24.6)	1.18 (0.59–2.37)
Abdominal pain	18/61 (29.5)	53/191 (27.7)	1.09 (0.55–2.15)
Asymptomatic carriage	12/61 (19.7)	19/191 (9.9)	2.22 (0.94–5.21)
Underlying disease	10/61 (16.4)	49/191 (25.7)	0.57 (0.25–1.27)
Treatment			
Antimicrobial agents	16/61 (26.2)	79/191 (41.4)	0.50 (0.25–1.00)
Intravenous fluid therapy	1/61 (1.6)	8/191 (4.2)	0.38 (0.38–3.05)
Outcome			
Relapse	4/61 (6.6)	6/191 (3.1)	2.16 (0.49–9.06)

[a]OR, odds ratio; CI, confidence interval.

stools, respectively. Among the nondiarrheic stool specimens, we isolated *A. butzleri* and *C. jejuni* in 7 (0.06%) and 205 (1.65%) stools, respectively. *A. butzleri* was more frequently isolated from diarrheic stool specimens than from nondiarrheic stools specimens. This difference was significant (OR 2.48, 95% CI 1.10–5.86) (p = 0.0175). We observed a similar result for the recovery of *C. jejuni* from diarrheic stool specimens compared with nondiarrheic stools specimens. This difference was even more significant (OR 3.68, 95% CI 3.18–4.25) (p < 0.0001).

Because medical records were unavailable for 6 patients with *A. butzleri* and 10 patients with *C. jejuni*, we compared the clinical features of 61 patients with *A. butzleri* matched against those of 191 patients with *C. jejuni*. Although diarrhea was a common clinical feature of both groups, it was more frequent in the *C. jejuni*–infected patients. The characteristics of the diarrhea differed significantly, however. Patients with *A. butzleri* were more likely to have persistent diarrhea (OR 3.97, 95% CI 1.4–11.3), or watery diarrhea (OR 2.20, 95% CI 1.18–4.13) than those with *C. jejuni* infection, but they were less likely to have acute diarrhea (OR 0.38 95%, CI 0.20–0.79). Other clinical features did not differ significantly. Asymptomatic carriage was more frequently encountered in patients with *A. butzleri* infection than in those with *C. jejuni* infection, but not significantly (OR 2.22, 95% CI 0.94–5.21).

Proportionally more patients with *C. jejuni* infections were treated with antimicrobial agents (41.4%) than patients with *A. butzleri* infections (26.2%) (OR 0.50, 95% CI 0.25–1.0). Among them, only 43.8% of patients with *A. butzleri* infections were treated empirically with an antimicrobial agent for which the strain was susceptible, whereas 79.5% of patients with *C. jejuni* received an appropriate antimicrobial drug.

Conclusions

In this study, *Arcobacter* was the fourth most common *Campylobacter* or *Campylobacter*-like organism isolated from stool specimens in our laboratories. Our observations suggest that *A. butzleri* display similar microbiologic and clinical features as *C. jejuni*. However, compared with *C. jejuni*, *A. butzleri* are more frequently associated with a persistent and watery diarrhea and less associated with bloody diarrhea. This first study on *Arcobacter* in humans could be the beginning of future research to better understand the pathogenesis and epidemiology of these non–*jejuni/coli Campylobacter.*

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Mycobacterium goodii Infections Associated with Surgical Implants at Colorado Hospital

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From February to October 2003, *Mycobacterium goodii* wound infections were identified among three patients who received surgical implants at a Colorado hospital. This report summarizes the investigation of the first reported nosocomial outbreak of *M. goodii*. Increased awareness is needed about the potential for nontuberculous mycobacteria to cause postoperative wound infections.

Mycobacterium goodii is a recently identified, rapidly growing nontuberculous mycobacteria species of the *M. smegmatis* group (1). Previously associated with sporadic cases of cellulitis, osteomyelitis, infected pacemaker sites, lipoid pneumonia (1,2), and bursitis (3), *M. goodii* has not been associated with outbreaks.

The Study

In June 2003, staff at hospital A contacted the Centers for Disease Control and Prevention (CDC) and the Colorado Department of Public Health and Environment (CDPHE) about two patients (patients 2 and 3) in whom *M. goodii* wound infections developed after surgery at hospital A. In October 2003, patient 1 notified CDPHE that he had a *M. goodii* wound infection; he had previously undergone surgery at hospital A.

Patient 1

A 64-year-old man underwent left total hip arthroplasty at hospital A in April 2002. In May 2003, he was evaluated at hospital A for chronic left hip pain. Bone scan suggested prosthesis loosening or infection. Gram stain and bacterial culture of aspirated joint fluid were negative. Mycobacterial culture was not performed.

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In July 2003, he underwent arthroplasty revision at hospital B for persistent pain. A preoperative sedimentation rate was normal, but a substantial amount of yellow fluid was noted during operative manipulation of the trochanteric bursa. Fluid Gram stain demonstrated a moderate number of leukocytes without organisms; bacterial culture was negative, and mycobacterial culture was not performed.

Wound erythema with drainage developed 4 weeks later, and septic hip arthritis was diagnosed at hospital B, requiring prosthesis removal. Joint fluid, decontaminated with Sputagest 50 mucolytic agent (Remel, Lenexa, KS), grew mycobacteria after 7 days of incubation at 36°C with Middlebrook 7H12 TB media (Becton Dickinson, Sparks, MD) and Lowenstein-Jensen agar. Resulting colonies were smooth, flat, mucoid, brownish-orange and later identified as *M. goodii* sensitive to ciprofloxacin (MIC= 1 μg/mL), doxycycline (MIC < 0.5 μg/mL), and trimethoprim/sulfamethoxazole (MIC= 1 μg/mL).

Patient 2

A 64-year-old man underwent right inguinal hernia repair with a Kugel patch at hospital A in January 2003. On postoperative day 17, he underwent elective L3–L4 laminectomy at hospital C. Right inguinal pain, swelling, and erythema developed 2 days later, and he required wound debridement and patch removal. Patch cultures grew mycobacteria after 3 days of incubation at 35°C with sheep blood agar and thioglycolate broth without pretreatment. The isolate was later identified as *M. goodii* sensitive to ciprofloxacin (MIC = 0.25 μg/mL), doxycycline (MIC < 0.5 μg/mL), and trimethoprim/sulfamethoxazole (MIC = 2 μg/mL).

Patient 3

A woman 75 years of age underwent a total replacement of her left knee at hospital A in April 2003. On postoperative day 14, pain and swelling developed in her left knee. Fluid aspirated from the wound on postoperative day 29 was negative by Gram stain and bacterial culture. Mycobacterial culture was not performed, and antimicrobial drugs were withheld. Repeat Gram stain 9 days later demonstrated many leukocytes without organisms. The prosthesis was later removed and replaced with a stabilizing polyethylene insert. Wound drainage samples, decontaminated with N-acetyl-L-cysteine-sodium hydroxide, grew mycobacteria after 11 days of incubation at 37°C with BacT/ALERT media (bioMérieux, Durham, NC) and Lowenstein-Jensen agar. The isolate was later identified as *M. goodii* sensitive to ciprofloxacin (MIC = 0.25 μg/mL), doxycycline (MIC < 0.5 μg/mL), and trimethoprim/sulfamethoxazole (MIC = 4 μg/mL).

Isolates were confirmed as *M. goodii* with 65-kD heat shock protein gene polymerase chain reaction restriction analysis, by using enzymes *Bst*EII, *Hae*III, *Bsa*HI, and *Aci*I (New England Biolabs, Beverly, MA) and matching them to a control strain, *M. goodii* ATCC 700504 (1). Susceptibilities were assessed according to the NCCLS document M24-A (4). Isolates were grown and compared by pulsed-field gel electrophoresis (PFGE) according to previously described methods (5). Genomic DNA restriction was performed with 40 U of *Ase*I (New England Biolabs, Beverly, MA). Less than a three-band difference was found among the resulting patterns (Figure). Based on previously described criteria (6), these isolates were closely related and probably from the same source.

Anesthesiologist A was the only person who had contact with all three patients during an operative procedure (Table). Anesthesiologist A was also present during an operation on a potential case-patient in whom an inguinal wound infection developed 29 days after his hernia had been repaired with a Kugel patch at hospital A in January 2003. Although mycobacterial cultures were not performed, a Gram stain of drainage material showed many leukocytes without organisms, and bacterial cultures were negative. This patient improved after surgical drainage, patch removal, and treatment with cephalexin. No other potential cases were identified retrospectively through questioning of surgical staff or prospectively with active surveillance.

Anesthesiologist A was assessed for *M. goodii* carriage by collecting a sputum sample, beard clippings, and sampling his nares and operating shoes with sterile swabs. Swabs were placed in Butterfield buffer (Becton Dickinson, Cockeysville, MD), exposed to 0.005% cetylpyridinium chloride monohydrate, vortexed, concentrated, added to 7H10 agar plates, and incubated at 35°C and 30°C for 14 days. Hand screening was also performed by using sterile premoistened wipes to wipe both hands and each finger. Wipes were processed and cultured similar to the procedure described above for swabs. All specimens from anesthesiologist A were negative.

Specimens from hands and operating room shoes were also collected from 12 staff members present in operating rooms before or during any of the three patients' procedures. All of these specimens were negative.

Water was tested by using sterile swabs to sample biofilm and water from faucets in the operating room area, including the scrub, sterile supply, and clean and dirty utility room sinks. Sterile swabs were also used to sample lights, vents, and anesthesia tables in the operating rooms, equipment sterilizers, and rubber floor mats adjacent to scrub sinks. All environmental samples were negative. No environmental breaches were found in the sterile supply area, including no evidence of water damage, and no recent construction.

Two patients received local anesthesia from single dose vials during surgery. Alcohol and povidone-iodine were used for skin preparation before all three procedures, and quaternary ammonium was used to disinfect operating rooms at hospital A. Clinical laboratories in Colorado were contacted, and no other *M. goodii* cases were identified in Colorado from 2002 to 2003.

Conclusions

Although this cluster occurred over an extensive period, it likely had a common etiology because *M. goodii* has been infrequently identified as a pathogen, and isolates had closely-related PFGE patterns. PFGE has been reported as a means of identifying outbreaks of other nontuberculous mycobacteria, including *M. fortuitum* (5), *M. chelonae*, and *M. abscessus* (7).

Additionally, all patients received surgical implants at the same hospital before their infections. Although patient 1 had arthroplasty revision at another hospital 1 month before diagnosis, chronic hip pain, an abnormal bone scan before revision, and an abnormal amount of bursal fluid during surgery suggest that he had indolent infection that preceded the second hip surgery.

Anesthesiologist A seemed a possible infection source because he was the only person present during each surgery at hospital A. Unscrubbed surgical personnel have been linked to other postoperative wound infections, including an anesthesiologist colonized with *Nocardia farcinica* who

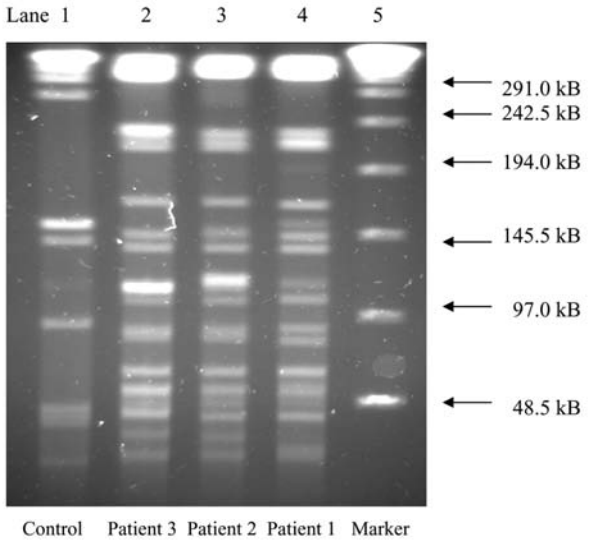

Figure. Pulsed-field gel electrophoresis patterns using restriction enzyme *Ase*I, of *Mycobacterium goodii* isolates from three patients with postoperative wound infections after receiving surgical implants from a hospital in Colorado. Lane 1 is control strain *M. goodii* ATCC 700504. Lanes 2–4 are case-isolates. Lane 5 is a 48.5-kb DNA marker.

Table. Summary of investigation of three cases of *Mycobacterium goodii* postoperative wound infections associated with surgical implants at a Colorado hospital

Criteria	Patient 1	Patient 2	Patient 3
Age	64	64	75
Sex	Male	Male	Female
Date of surgery	4/02	1/03	4/03
Date infection diagnosed	8/03 (16 mo after surgery)	2/03 (19 d after surgery)	5/03 (29 d after surgery)
Initial surgery	Total hip arthroplasty	Inguinal hernia repair	Total knee replacement
Hospital[a]	A	A	A
Surgical implant	Artificial hip	Kugel patch	Artificial knee
Operating room (OR)[b]	A	B	A
Surgeon[c]	A	B	C
Anesthesiologist[d]	A	A	A
Assistant[e]	A	None	A, B
OR personnel[f]	A, B, C, D	A, E	B, F, G, H
Skin preparation	Povidone-iodine, isopropyl alcohol	Povidone-iodine, ethanol	Povidone-iodine, isopropyl alcohol
Local anesthetic	Bupivicaine	Bupivicaine	None
Saline irrigation	Yes (with kanamycin)	Yes	Yes
Intraoperative antibiotics	Cefazolin	None	Cefazolin
Notes	Hip aspiration, hospital A, 5/03; Hip arthroplasty revision, hospital B, 7/03	Laminectomy, hospital C, 2/03	

[a]Includes circulating nurse, scrub technician, surgical sales representative, and any staff not previously listed who were working in the operating room.
[a]The three patients received implants at hospital A.
[b]The three patients had surgery in two operating rooms (A and B) at hospital A.
[c]Three surgeons (A–C) were involved in these three operations at hospital A.
[d]One anesthesiologist (A) was involved in the three operations at hospital A.
[e]Two different assistants (A and B) were involved in two of the three operations at hospital A.
[f]Includes circulating nurses, scrub technicians, a surgical sales representative and any staff not previously listed who were working in the operating rooms during these operations (A–H).

reportedly caused five postoperative sternotomy infections (8). Although our investigation did not identify anesthesiologist A as an *M. goodii* carrier, carriage status was assessed months after the outbreak, and transient carriage is possible. Additionally, we were unable to identify published methods on how to assess *M. goodii* carriage.

M. goodii was not isolated from hospital A's water supply, based on swabs of biofilm and water from sinks, but other outbreaks of nontuberculous mycobacteria infections have been linked to municipal water (9). In one study, 95 (83%) of 115 dialysis centers had nontuberculous mycobacteria in their water supply (10). Many nontuberculous mycobacteria grow in biofilms that can form at faucet outlets, and several grow in hot, chlorinated, and distilled water (9). Although *M. goodii* was not isolated from any sink or water specimens, a water source could not definitively be ruled out. The hospital superheats the water system annually to 87.2°C (last done 1 month before this investigation) but does not culture water specimens. Therefore, water flora at the time of these infections could have been different from the flora present during the investigation. Additionally, because water specimens were collected with swabs rather than through a bulk water collection, *M. goodii* contamination might have been missed if concentrations were sufficiently low.

Although multidose vials were not linked to this outbreak, they caused other nontuberculous mycobacteria outbreaks (9,11). Several nontuberculous mycobacteria are resistant to mercury, which is commonly used as a preservative in multidose vials (9).

Nontuberculous mycobacteria are relatively resistant to disinfectants (9), and disinfectant and antiseptic effectiveness against *M. goodii* have not been reported. *M. smegmatis*, which is closely related to *M. goodii*, is sensitive to alkaline glutaraldehyde (12,13), povidone iodine (13,14), and chlorhexidene gluconate disinfectants. Sodium hypochlorite, ethanol, phenol, and quaternary ammonium are less effective (13).

M. goodii is generally susceptible to amikacin, ethambutol, and sulfamethoxazole. It has intermediate susceptibility to ciprofloxacin, doxycycline, and tobramycin; variable susceptibility to cefmetazole, cefoxitin, and clarithromycin; and resistance to isoniazid and rifampin (1). For mild wound infections, monotherapy with an oral agent for 4–6 months has been effective. In more severe disease, surgical debridement, initial combination therapy, followed by oral therapy, to complete 6 months of treatment has been effective (2).

The lack of an identified infection source made focused control measures difficult. Measures implemented by hospital A included requiring single-dose medication vials for invasive procedures; culturing for mycobacteria in wound cultures with negative bacterial growth; requiring staff to cover scrubs and remove shoe

and hair covers when leaving the operating room area and to apply new covers upon reentering; requiring staff to use dedicated shoes in the operating room; having patients take preoperative showers with antiseptic soap; using tobramycin-impregnated cement in appropriate procedures; avoiding flash sterilization of implantable devices; and using phenol disinfectant weekly and quaternary ammonium disinfectant daily to disinfect operating rooms. Although data for *M. smegmatis* suggest that phenol and quaternary ammonium disinfectants might not be effective against *M. goodii* (13), evidence is insufficient to make firm recommendations.

Patients 2 and 3 received a diagnosis relatively quickly, but patient 1 had a prolonged, indolent infection and had an arthroplasty revision before diagnosis. Several wound cultures were performed without assessment for mycobacterial infection. Although documented (9), nontuberculous mycobacteria wound infections are less common than bacterial infections, and clinicians might be less aware of nontuberculous mycobacteria as potential causes of surgical site infections.

Research is needed to determine effective antiseptics and disinfectants against *M. goodii*. Clinicians should be aware of the association of nontuberculous mycobacteria with surgical site infections and consider testing for mycobacteria when a Gram stain shows notable numbers of leukocytes, but wound cultures are negative for bacteria.

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Dr. Ferguson is a family physician and Epidemic Intelligence Service officer, CDC, assigned to the Colorado Department of Public Health and Environment. He is particularly interested in outbreak investigations of infectious diseases.

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Nosocomial Acquisition of Dengue

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Recent transmission of dengue viruses has increased in tropical and subtropical areas and in industrialized countries because of international travel. We describe a case of nosocomial transmission of dengue virus in Germany by a needlestick injury. Diagnosis was made by TaqMan reverse transcription–polymerase chain reaction when serologic studies were negative.

Dengue viruses are transmitted by *Aedes* mosquitoes in tropical regions worldwide. The global incidence of epidemic and endemic dengue fever has increased substantially and is estimated at 50–100 million cases per year. International travel leads to imported cases in countries of the Northern Hemisphere (1), where dengue fever is an important differential diagnosis of fever in travelers returning from the tropics. Occupational needlestick injuries continue to pose a substantial risk for healthcare workers and occur at rates of 1.0 to 6.2 per 100 person-years (2). Common concerns are the transmission of HIV, hepatitis B virus, and hepatitis C virus. However, other pathogens can be transmitted as well. We report a case of nosocomial transmission of dengue virus.

The Study

The index patient, a 26-year-old woman, was admitted to the infectious disease ward of a university hospital with a temperature of 40°C and myalgias 3 days after she returned from a 3-week trip to Cambodia and Thailand. Dengue virus infection was subsequently diagnosed, and mild hepatitis and a rash developed. She was discharged in good condition after the fever subsided. On the day of admission of the index patient (day 0_i), a nurse sustained a needlestick injury with a hollow needle that had been used for drawing blood from the index patient. The needlestick resulted in a bleeding puncture wound that was immediately treated with an antiseptic. The index patient did not report any high-risk activity for HIV or hepatitis B virus, and the nurse had been immunized against hepatitis B virus. Therefore, no specific postexposure prophylaxis was performed. The nurse had previously been in good health and had not traveled outside Germany in the preceding 12 months.

Four days after the needlestick, headache, myalgias, and arthralgias developed in the healthcare worker, for which she took ibuprofen. Seven days later, when she was experiencing an intense headache and noticed a macular rash on her trunk, she sought treatment from a local doctor (day 0_n). Physical examination showed bilateral cervical lymphadenopathy. On day 2_n, she visited our service, where dengue virus infection was diagnosed by using a Light Cycler (Roche Diagnostics, Mannheim, Germany) polymerase chain reaction (PCR) method. Her symptoms lessened gradually over the course of 4 weeks, and she was on sick leave for 5 weeks. The time frame of the respective clinical presentation and the virologic results of the index patient and the nurse are shown in the Figure; laboratory data are presented in the Table.

Serologic studies were performed with the PanBio dengue immunoglobulin (Ig) M capture enzyme-linked immunosorbent assay (ELISA) and PanBio dengue indirect IgG ELISA (PanBio Ltd., Brisbane, Australia) (3); arbitrary units relative to a simultaneously measured calibrator >11 were considered positive. For detecting virus RNA, RNA was prepared from 140 µL of serum by using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

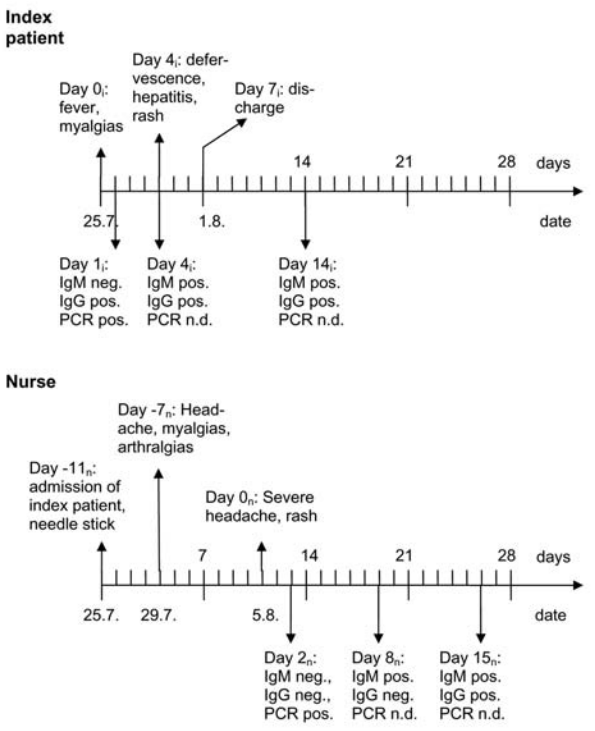

Figure. Time line of the signs, symptoms, and diagnostic tests in the index patient (i) and nurse (n). Ig, immunoglobulin; PCR, polymerase chain reaction; n.d., not done.

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Table. Laboratory data for index patient and health care worker infected with dengue virus[a]

	Day	Leukocytes (μL)	Lymphocytes (μL)	Thrombocytes (μL)	Dengue IgM EIA	Dengue IgG EIA (U)	Dengue PCR
Index patient	0_i	2.000	420	137.000	–	+ (16.4)	+
	4_i	1.900	ND	55.000	+	+ (43.0)	–
	14_i	5.000	1.650	375.000	+	+ (30.9)	ND
Health care worker	2_n	2.600	590	136.000	–	– (2.8)	+
	8_n	4.000	1.040	174.000	+	– (5.9)	ND
	15_n	4.200	1.190	213.000	+	+ (16.4)	ND
	22_n	4.800	1.220	215.000	+	+ (21.7)	ND

[a]Ig, immunoglobulin; EIA, enyzyme immunosorbent assay; PCR, polymerase chain reaction; ND, not done.

To detect specific dengue virus RNA, we adapted a TaqMan-reverse transcription (RT)-PCR (4) to detect any of the four serotypes by using the following: degenerated forward primer (DEN FP), reverse primer (DEN RP); and probe (DEN P): DEN FP 5′AAggACTAgAgg TTAKAggAgACCC3′, DEN RP 5′ggCCYTCTgTgC CTggAWTgATg3′ and the probe DEN P 5′ FAM-AACAgCATATTgACgCTgggARAgACC-TAMRA-3′. RT-PCR conditions for the Light Cycler (Roche Diagnostics) were: RT at 61°C for 20 min, activation at 95°C for 5 min, and 40 cycles of PCR at 95°C for 15 s, 60°C for 60 s. We used the RNA Master Hybridization Probes Kit (Roche Diagnostics) with 500-nM primers and 200-nM probes. The kit includes an aptamer-blocked *Thermus thermophilus* DNA polymerase, which performs RT and, once the aptamer drops out at activation, hotstarts PCR amplification.

Conclusions

This is the fourth reported case, to our knowledge, of nosocomial dengue virus transmission (5–7) and the first in which TaqMan RT-PCR was used to provide evidence of nosocomial transmission before the detection of an antibody response. The index patient had acquired a dengue virus infection in Southeast Asia and experienced typical symptoms. In particular, she was febrile on admission, when the needlestick injury of the nurse occurred. In the health care worker who sustained the injury, cephalgia and myalgias developed after an incubation period of 4 days. A typical rash appeared after 11 days, when she also had a severe headache. The absence of fever, the most common sign of dengue fever, is likely due to the administration of ibuprofen. Both persons completely recovered. However, the healthcare worker was on sick leave for 5 weeks with resulting socioeconomic consequences.

The diagnosis was confirmed in both cases by both seroconversion and detection of dengue viral RNA by TaqMan RT-PCR; the latter gave positive results in both cases 3 and 6 days, respectively, before serum specimens were shown to contain antibody. Dengue viremia is known to correlate well with the presence of fever (8), which was the case in the index patient. Our report illustrates the potential of percutaneous nosocomial transmission of dengue viruses. This risk is likely to increase with the increase in the number of dengue infections imported to countries where dengue viruses are not endemic.

Dr. Wagner is an infectious disease specialist at the University Hospital, Freiburg, Germany. His research interests focus on iron metabolism and intracellular survival of mycobacteria.

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Novel Recombinant Sapovirus

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We determined the complete genome sequences of two sapovirus strains isolated in Thailand and Japan. One of these strains represented a novel, naturally occurring recombinant sapovirus. Evidence suggested the recombination site was at the polymerase-capsid junction within open reading frame one.

The positive-sense polyadenylated single-stranded RNA virus family *Caliciviridae* contains four genera, *Norovirus*, *Sapovirus*, *Lagovirus*, and *Vesivirus* (1). Human norovirus is the most important cause of outbreaks of gastroenteritis in the United States and infects all age groups (2). Human sapovirus is also a causative agent of gastroenteritis but is more frequent in young children than in adults (3). Most animal caliciviruses are grouped within the other two genera. In 1999, Jiang et al. (4) identified the first naturally occurring human recombinant norovirus, and several other strains were later described as recombinants (5–8). Evidence suggested that the recombination event occurred at the junction of open reading frames one and two (ORF1 and ORF2), but this finding was not proven. Norovirus ORF1 encodes nonstructural proteins, including the RNA-dependent RNA polymerase, ORF2 encodes the capsid protein, and ORF3 encodes a small capsid protein (1). Nucleotide sequence of the polymerase and capsid junction generally is conserved among the human norovirus genotypes (4,6), which likely facilitates a recombination event when nucleic acid sequences of parental strains come into physical contact in infected cells, e.g., during copy choice recombination (9).

The Study

We used genetic analysis to investigate a novel, naturally occurring recombinant sapovirus. Two strains were used for the analysis, Mc10 strain (GenBank accession no. AY237420), isolated from an infant hospitalized with acute gastroenteritis in Chiang Mai, Thailand, in 2000 (5), and C12 strain (AY603425), isolated from an infant with gastroenteritis in Sakai, Japan, in 2001 (unpub. data). Although the original polymerase chain reaction (PCR) primer sets that detected these two strains were different, both were directed toward the conserved 5′ end of the capsid gene and have been shown to detect a broad range of sapovirus sequences in genogroup I (GI) and GII (5,10). For Mc10, primers SV5317 and SV5749 were used; for C12, primers SV-F11 and SV-R1 were used.

The complete genomes for Mc10 and C12 were determined as previously described (6). As shown in Figure 1A, the sapovirus genome has an organization slightly different from that of the norovirus genome. ORF1 encodes nonstructural proteins, polymerase, and the capsid protein, and ORF2 encodes a small protein (1).

Initially, we grouped Mc10 and C12 into two distinct GII clusters (i.e., genotypes), on the basis of their capsid sequences (Figure 2A) and the phylogenetic classification scheme of Okada et al. (10). In addition, the overall genomic nucleotide similarity between Mc10 and C12 was 84.3%, while ORF1 and ORF2 shared 85.5% and 73.3% nucleotide identity, respectively. These results corresponded with the capsid-based grouping shown in Figure 2A. By comparing sequence similarity across the length of the genomes with SimPlot with a window size of 100 (11), we discovered a potential recombination site, where the similarity analysis showed a sudden drop in nucleotide identity after the polymerase region (Figure 1B). Nucleotide sequence analysis of ORF1 less the capsid sequence and the capsid sequence indicated 90.1% and 71.3% nucleotide identity, respectively (Figure 1A). To additionally illustrate the nucleotide identities of ORF1 less the capsid sequence, a phylogenetic tree of polymerase sequences of Mc10, C12, and other available strains was developed (Figure 2B). However, for three strains (Mex14917/00,

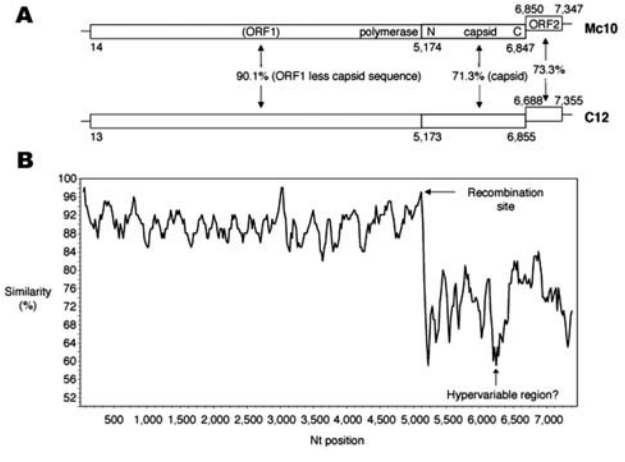

Figure 1. A) The genomic organization of Mc10 and C12 strains. B) the SimPlot analysis of Mc10 and C12. Mc10 genome sequence was compared to C12 by using a window size of 100 bp with an increment of 20 bp. All gaps were removed. The recombination site is suspected to be located between polymerase and capsid genes, as shown by the arrow. The possible hypervariable region for the capsid protein is also shown.

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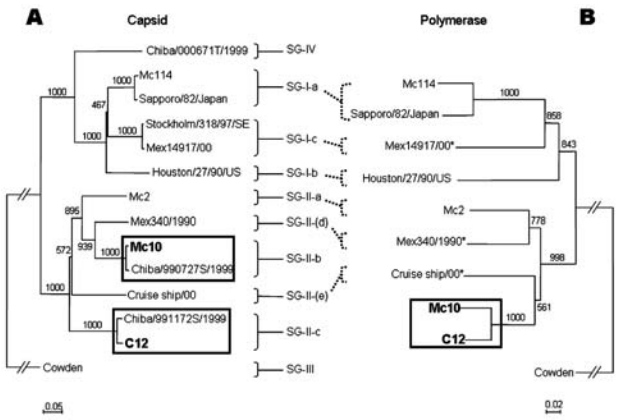

Figure 2. Phylogenetic analysis of (A) capsid (376 nt) and (B) polymerase (289 nt) sequences of Mc10, C12, and additional strains in GenBank. Sapovirus capsid sequences were classified on the basis of the scheme of Okada et al. (10). Two unclassified strains, Mex340/1990 and Cruise ship/00, were assigned SG-II-(d) and SG-II-(e). The asterisks indicate noncontinuous polymerase-capsid sequences. The numbers on each branch indicate the bootstrap values for the genotype. Bootstrap values of >950 were considered statistically significant for the grouping (6). The scale represents nucleotide substitutions per site. GenBank accession no. for the reference strains are as follows: Chiba/000671T/1999, AJ412805; Chiba/990727S/1999, AJ412795; Chiba/991172S/1999, AJ412797; Mc114, AY237422; Cruise ship/00, AY289804 and AY157863; Cowden, AF182760; Houston/27/90/US, U95644; Mc2, AY237419; Mc10, AY237420; Mex340/1990, AF435809 and AF435812; Mex14917/00, AF435813 and AF35810; Sapporo/82/Japan, U65427; and Stockholm/318/97/SE, AF194182.

Mex340/1990, and Cruise ship/00), the polymerase and capsid sequences of ORF1 were not continuous, i.e., they may represent two different strains. Nevertheless, Mc10 and C12 were in the same cluster by polymerase-based grouping but were in distinct clusters by capsid-based grouping (Figure 2). All other strains maintained clusters by polymerase- and capsid-based groupings.

These findings showed Mc10 and C12 had high sequence identity up to the beginning of the capsid region where the sequence identity was considerably lower. These results are easily explained by a recombination event, a single point recombination event occurring at the polymerase-capsid junction. At the end of the polymerase region, there were 44 nt, which included the first 8 nt of the capsid gene and showed 100% homology. After these nucleotides, the identity decreased and was clearly different, as shown in Figure 1B. This conserved region may represent the break and rejoin site for Mc10 and C12 during viral replication, although direct evidence for this event is lacking.

A sudden drop was indicated, followed by a rise in nucleotide identity between nt 6,250 and 6,500 (Figure 1B). Although our initial hypothesis was that another recombination event occurred, closer inspection indicated that this region corresponded to amino acids 358 and 440 for the capsid protein and likely represented the hypervariable region, as described recently in the structural analysis of sapovirus capsid protein (12). For recombinant norovirus strains, we also observed a sudden decrease in nucleotide identity in the related capsid region (13), which represents the outermost protruding domain (P2) and is subject to immune pressure (14). For these reasons, a low homology, even between closely related strains, is generally seen in this region (6), although further studies by sequence analysis with other strains are needed.

In a recent study, we genetically and antigenically analyzed two recombinant norovirus strains (13). When the polymerase-based grouping was performed, these two strains clustered together; when capsid-based grouping was performed, these two strains belonged in two distinct genotypes. When we compared the cross-reactivity of these two viruslike particles (VLPs) and hyperimmune sera against the VLPs, we found distinct antigenic types for the VLPs, although a considerable level of cross-reactivity was found between them. We recently expressed C12 capsid protein that resulted in the formation of VLPs, but we were unsuccessful in expressing Mc10 VLPs (G.S. Hansman, unpub. data); therefore the antigenicity of these two strains remains unknown.

Jiang et al. (4) reported two potential parental norovirus strains that were cocirculating in the same geographic region (Mendoza, Argentina, in 1995), which provides some evidence for where and when the recombination event may have occurred. In addition, Jiang identified the progeny strain from the event, the Arg320 strain. In our study, Mc10 and C12 were isolated from Thailand and Japan, respectively, but we have no evidence for the place and time of the event. While the genetic analysis for Mc10 and C12 identified a possible recombinant sapovirus strain, the analysis does not clarify which of the two strains was the parent strain and which was the progeny strain. Further extensive studies are needed that perform sequence analysis of polymerase and capsid genes and compare results with analysis of other strains. Nevertheless, other strains with capsid sequences that closely match those of Mc10 and C12 are in the public database, which suggests the circulation of other recombinant sapovirus strains.

Conclusions

Recombination and evolution are important survival events for all living creatures as well as viruses. These events in viruses are not completely understood, but they can be potentially dangerous for host species, and they likely influence vaccine designs (15). From our studies, the human sapovirus and norovirus recombination appears limited to the intragenogroup because no intergenogroup

or intergenus recombination has yet been identified and recombination only occurs at the polymerase-capsid junction. Finally, the results of this study have increased our awareness of recombination in the *Sapovirus* genus and may have an influence on the future phylogenetic classification of sapovirus strains.

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SARS Patients and Need for Treatment

To the Editor: We read with interest the case report by Wong et al. (1). Three similar cases of serologically confirmed severe acute respiratory syndrome (SARS) were treated in our hospital; all of the patients recovered uneventfully without specific treatment. They had either negative results on polymerase chain reaction (PCR) tests for SARS-associated coronavirus (SAR-CoV), or they were admitted when such rapid diagnostic tests were not yet available; hence, SARS-specific treatment was not prescribed.

The first patient was a 35-year-old previously healthy female tourist from Guangzhou, China, who was admitted to our hospital in late February 2003. She had visited several family members who had atypical pneumonia; some eventually died from the disease. The patient had fever, chills, and dry cough approximately l week after exposure but experienced no mylagia, diarrhea, or shortness of breath. On physical examination, scanty crepitations were heard in her right lower chest, and chest radiographs showed right lower zone consolidation. Blood tests showed a slightly low platelet count of 119 x 10^9/L and mildly elevated alanine transaminase at 59 U/L (normal <55 U/L), but total and differential leukocyte counts were normal. Tests for etiologic agents included blood and sputum bacterial cultures; sputum for acid-fast bacilli; and nasopharyngeal aspirates for influenza, parainfluenza, adenovirus, and respiratory syncytical virus. Serologic titers for *Mycoplasma*, C*hlamydia psittaci*, and *Legionella* were negative. Reverse transcription (RT)-PCR tests for SARS-CoV were not available at that time.

Oral clarithromycin and intravenous amoxillin-clavulanate (subsequntly switched to levofloxacin) were prescribed. Her high fever (temperature 39.5°C) lasted for 4 days and then gradually subsided; the radiologic abnormality also improved progressively after the first week. Oxygen supplementation of 2 L/min was necessary for the first 2 days. The diagnosis of SARS was made when the patient's convalescent-phase serum sample, collected 33 days after discharge (day 45 of illness), showed an elevated anti-SARS immunoglobulin (Ig) G titer of 1:800 by immunofluorescence.

The second patient was a 34-year-old previously healthy man; his father had shared a hospital cubicle with a patient who was subsequently diagnosed with SARS. Fever (temperature 39°C), chills, and rigors developed in patient 2 on December 3, 2003, approximately 4 days after his first hospital visit to his father; he had no cough or gastrointestinal symptoms. Chest radiographs showed right lower zone consolidation. Blood tests showed low platelet count of 91 x 10^9/L, elevated creatinine kinase (370 U/L), and elevated lactate dehydrogenase levels (1,060 U/L). Total and differential leukocyte counts were normal. Tests for etiologic agents of pneumonia had negative results. His fever (the highest temperature was 39.5°C) subsided after day 2 of admission, with a transient spike on day 11 that coincided with a slight increase in right lower zone consolidation. Both abnormalities subsequently resolved promptly, and no oxygen supplement was necessary. RT-PCR test for SARS-CoV was not available in our hospital when he was admitted. However, SARS was diagnosed when his convalescent-phase serum, collected on day 21 of illness, demonstrated a SARS-CoV IgG titer (by immunofluorescence) of 1:3,200, from an initial baseline of <1:25, taken on day 12 of his illness. No treatment was given, since the patient had already fully recovered when the results arrived.

The last patient was a 74-year-old, previously healthy man, who had visited a sick relative; the relative was later diagnosed with SARS. Fever, chills, and cough developed in our patient 4 days later. Chest radiograph showed left lower and middle zone consolidations. Intravenous ceftriaxone and oral clarithromycin were started. Blood tests showed elevated alkaline phosphatase of 226 U/L and alanine transaminase of 126 U/L. His initial leukocyte count was 18.8 x 10^9/L with neutrophilia (16 x 10^9/L, 85.2%) and a normal lymphocyte count of 1.2 x 10^9/L; platelet count was normal. No causative agent was identified, including by RT-PCR test for SARS-CoV. His fever had subsided upon admission, and serial chest radiographs, liver function, and leukocyte counts showed progressive improvement without specific treatment. The diagnosis of SARS was made from two elevated SARS-CoV IgG levels of both 1:3,200 (by immunofluorescence), taken at days 5 and 24 after his admission (days 19 and 38 of illness).

Although SARS was diagnosed in these three patients retrospectively, and they were not treated with antiviral agents, they were managed in isolation wards. Patients reported adhering to droplet precautions after discharge (mainly, wearing surgical face masks when in close contact with others), and none was believed to have transmitted the virus to others.

SARS can be associated with a substantial death rate (2). Ribavirin and systemic corticosteroids were used in our hospital during the SARS epidemic. However, the efficacy of this regimen has not been proven, and concerns exist about side effects of both drugs (3,4). Some retrospective analyses suggested using lopinavir/ritonavir and integrative Chinese and Western medicine were associated with improved outcomes (5,6). In vitro (7,8) and animal (9) studies have suggested that interferon and monoclonal antibodies might have some effects on the disease. However, data

from randomized controlled trials are lacking. All of our patients had been previously healthy, with no coexisting conditions identified as poor prognostic risk factors (2,10). These three cases, together with the case of Wong et al. (1), suggested that at least a subset of SARS adult patients can have a relatively benign clinical course and uneventful recovery, without any specific treatment other than antimicrobial agents.

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Occupational Malaria Following Needlestick Injury

To the Editor: A 24-year-old female nurse was admitted to the emergency room at Bichat University Hospital in Paris, France, on July 4, 2001, with fever, nausea, and general malaise. She had no notable medical history, except spontaneously regressive Schönlein-Henloch purpura at 9 months of age. On admission, after she was given paracetamol, her axillary temperature was 37.6°C. She was slightly jaundiced and reported a mild headache but showed no resistance to head flexion. Her abdomen was depressible but tender. Urinalysis did not show hematuria or signs of urinary infection. Biologic tests indicated normal values except the following: platelets 47.4 x 10^3/µL, aspartate aminotransferase 307 U/L (normal value <56), alanine aminotransferase 239 U/L (normal value <56), total bilirubin 58 µmol/L (normal value <24), and γ-glutamyl transpeptidase 57 U/L (normal value <35). Results of an abdominal echogram were normal. Result of a blood film to identify *Plasmodium falciparum* was positive for parasitemia at 0.038 per 100 erythrocytes. The patient was given 500 mg of oral quinine three times daily; intravenous quinine was administered 15 hours after admission because she became nauseated. Her malaise persisted for 3 days, but she did not show any signs of malaria. She recovered completely and was discharged on day 6 of hospitalization.

The patient had not traveled outside France except to the United Kingdom years earlier. She did not live near an airport, nor had she been to one recently. She had vacationed in the south of France from June 23 to June 26 but had traveled by car. She had been certified as a registered nurse on May 28 and had been working as a substitute employee at various hospitals in the greater Paris area. On June 21, 2001, she sustained an accidental needlestick injury while taking a blood sample with an 18-gauge, peripheral venous catheter that had no safety feature. She removed the catheter stylet and stuck herself as she crossed her hands to discard the stylet in a sharps container. The needlestick pierced the nurse's glove and caused a deep, blood-letting injury on the anterior aspect of the left wrist. She had no previous history of needlestick injury. She notified the hospital occupational medicine department of her injury on the day it occurred and was given a postexposure interview. In accordance with national postexposure management guidelines, she was tested for HIV and hepatitis C virus (HCV) antibody, and results were negative at baseline; her immunization against hepatitis B virus (HBV) was confirmed. The risk of infection by pathogens other than HBV, HCV, or HIV following a needlestick injury was not discussed during her postexposure interview, and the nurse was not made aware of that risk. The injured nurse did not inform the managing physician that the injury had occurred while she was drawing

blood from a patient to determine if the patient was infected with malaria.

By July 1, 10 days after exposure, fatigue, malaise, and fever developed; her temperature was lowered to 38.6°C by taking paracetamol. Her condition returned to normal on July 2 before a second bout of fever and myalgia occurred during the night. She had to leave work early on July 3 because of generalized pain and a temperature of 39°C. The patient's mother is a biologist and was aware that her daughter had sustained a needlestick injury while drawing blood from a patient in whom malaria was suspected. The mother insisted that a blood smear be performed at a private laboratory in Paris. The smear was qualitatively determined positive for *P. vivax*. Subsequently, the patient was admitted to Bichat-Claude Bernard University Hospital with suspected malaria. A repeat blood smear conducted there identified *P. falciparum*.

The source patient was a 28-weeks' pregnant, 30-year-old woman of Kenyan origin who resided in France; she had visited Kenya and returned to France on June 1, 2001. On June 21, she was admitted to the gynecology-obstetrics emergency room at a greater Paris area hospital with fever and malaise. Blood sampling and thin and thick blood smears were performed by the nurse. The source patient's level of parasitemia was estimated at 0.05 per 100 erythrocytes, and oral quinine was initiated. The physician who interviewed the nurse after the needlestick injury verified that the source patient was HIV- and HCV-antibody negative and that the nurse was immunized against HBV. On June 23, although the results of her test for *Plasmodium* were negative, she was transferred to another tertiary care center where IV quinine was administered for nausea and vomiting, and she could be monitored more closely. She recovered fully and was discharged on June 27. Unfortunately, all blood samples or smears from the source patient had been discarded by the time the injured nurse became ill.

P. falciparum is a bloodborne pathogen, and malaria is a well-documented complication of transfusion (1). Malaria has also been diagnosed after intravenous drug use (2,3) and breaches in infection control procedures (4–6), as well as occupational exposures (1–5). Occupational *P. falciparum* infection after a needlestick injury may be rare; however, such an injury can be potentially severe in nonimmune healthcare workers in countries where malaria is not endemic, especially if the occupationally infected person is pregnant. This situation may also become more common as malaria spreads and as increasing international travel brings potential source patients to hospitals in malaria-endemic countries.

HBV, HCV, and HIV are the pathogens most often transmitted in documented cases of occupational infection following needlestick injuries in industrialized countries. Testing for infection by these pathogens does not include all the possible infections that can result from occupational exposure (1,7,8). Although conducting a thorough investigation of the circumstances surrounding any needlestick injury is a challenge in the daily clinical setting, an investigation should always be carried out. As in this case-patient, the treatment of occupational *P. falciparum* infection may be delayed because physicians do not immediately consider malaria as a possible diagnosis. Furthermore, healthcare workers with neurologic symptoms caused by *P. falciparum* malaria may be too ill to tell the treating physician about their occupational exposure. Such infections must be diagnosed promptly as they are potentially lethal, and presumptive treatment is readily available and well tolerated. Clinicians managing healthcare or laboratory workers with a febrile illness or in a postexposure setting should consider the probability of occupational *P. falciparum* malaria.

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Nosocomial Transmission of Dengue

To the Editor: Four viruses form the dengue complex of mosquito-borne viruses (family *Flaviviridae*, genus *Flavivirus*). Any of these viruses can cause dengue fever, an uncomplicated febrile illness with rash; however, these viruses are not transmitted person to person. The principal mosquito vector of these viruses is *Aedes aegypti*. These viruses are not known to exist in Europe; therefore, dengue virus infections in Europe are seen in patients returning from dengue-endemic areas (1). Nosocomial transmissions of dengue viruses by needlestick have been reported in three instances (2–4) and by bone marrow transplant in one instance (5). We describe the first case of nosocomial dengue fever diagnosed and treated in Hungary.

On September 6, 2003, a 46-year-old physician sought care from the Department of Infectology, ("Baranya County Hospital" Pécs, Hungary); he reported a 4-day history of fever, headache, malaise, maculopapular rash, and pharyngitis. He had recently returned from a trip to Thailand and recalled having been bitten by a mosquito at Bangkok airport 11 days earlier. The patient had no history of illnesses before he left Hungary to go to Thailand. On examination, laboratory results indicated leukopenia (3,300 leukocytes/mm^3) and mild thrombocytopenia (119,000 platelets/mm^3). Leukopenia is characteristic of dengue virus and has been associated with suppression of bone marrow production (6). We conducted additional tests because thrombocytopenia could have been the first sign of a more severe form of dengue infection, dengue hemorrhagic fever, which is associated with hemorrhagic diathesis and shock (6). Lymphocytosis and monocytosis with 26% atypical lymphocytes and a high-normal level of alanine aminotransferase (56 U/L) were found. The C-reactive protein level and the erythrocyte sedimentation rate were normal. Blood smears for malarial parasites were negative.

Examination of the patient showed a maculopapular rash, pharyngitis, and conjunctivitis. Dengue fever was the clinical diagnosis based on the patient's history of a mosquito bite in a dengue-endemic country, the patient's symptoms, and the laboratory results. The patient's general condition was relatively good, so we treated him on an outpatient basis and recommended that he return for daily examinations.

On September 7, while collecting a blood sample from the patient, the patient's sister, also a physician, accidentally stuck her finger with the needle, which was contaminated with the patient's blood. Seven days later she became ill, with fever, headache, diffuse maculopapular rash, myalgia, cervical lymphadenopathy, and malaise. Her laboratory tests showed leukopenia with a normal thrombocyte level, C-reactive protein level, liver function tests, and erythrocyte sedimentation rate. On physical examination, painfully enlarged cervical lymph nodes and conjunctivitis were found. No complications were observed and the disease resolved within 10 days after onset in both patients. The female patient had never traveled to a dengue-endemic region.

Serologic and virologic evidence confirmed the clinical diagnosis. Acute-phase serum samples from each patient were tested for immunoglobulin (Ig) M and IgG antibodies to dengue viruses by using a commercial enzyme-linked immunosorbent assay kit. IgM, but not IgG, antibodies to dengue viruses were detected in the serum sample from the male patient 7 days after the onset of his illness; a convalescent-phase serum sample was not available for further testing. The first serum sample was obtained from the female patient 6 days after onset of her illness. IgM and IgG antibodies were not found in that sample. In the serum sample obtained from the female patient 12 days after onset, IgM, but not IgG, antibodies to dengue viruses were found. Both IgM and IgG antibodies were found in serum samples from this patient 3 weeks after onset of her illness.

Diagnosis was also confirmed by reverse transcription–polymerase chain reaction assays of early serum samples of both patients by using universal flavivirus primers. Amplification products were directly sequenced (GenBank accession no. AY538627 and AY538628). The nucleotide sequences were identified with a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) using the GenBank database. Highest similarity was with dengue virus type 2 strain ThNH76/93, which had been isolated from a patient in northeast Thailand during the epidemic season of 1993 (7). The virus- specific nucleotide sequences detected in the Hungarian patients showed 98% nucleotide identity with the corresponding sequences of the Thai strain.

Viremia and simultaneous antibody production has been observed in several studies of dengue (6,8,9). Virus isolation is possible in dengue infections early in the illness, and in our experience, virus RNA was

detected during the early febrile period. The male patient still had fever when the needle accident occurred, and the needle was contaminated.

Infectious disease specialists and other physicians should recognize that vector-borne diseases, such as dengue and malaria, are potentially life threatening. Therefore, they should consider these diseases in the differential diagnosis of febrile patients returning from tropical countries. In most patients, dengue fever resolves without hemoconcentration, an indication of dengue hemorrhagic fever. Nosocomial transmission of dengue viruses is not a common event, however, physicians must consider these diseases.

These unique cases demonstrate the possible introduction and transmission of exotic tropical viruses in a country within temperate zones; all that is needed are competent vectors. Whereas *A. aegypti* is not endemic in Europe, it could be introduced. The *A. albopictus* mosquitoes, an invader from Asia, already exists there, albeit in isolated areas (10). Patients returning from distant regions should be treated with increased attention and care. Although dengue viruses are rarely transmitted person to person, this incident emphasizes the importance of having reliable and rapid diagnostic methods available for early detection of imported infections with exotic viral agents.

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Human Crimean-Congo Hemorrhagic Fever, Sénégal

To the Editor: Crimean-Congo hemorrhagic fever (CCHF) virus, genus *Nairovirus*, family *Bunyviridae*, is transmitted to mammals and birds by ticks. *Hyalomma* ticks, the primary vectors in CCHF transmission, are widespread throughout Europe, Asia, the Middle East, and Africa; evidence of CCHF virus has been found in all these regions. CCHF in humans is an acute viral disease that is transmitted by the bite of infected ticks, direct contact with blood or infected tissues from viremic animals, and direct contact with the blood or secretions of an infected person (1).

On January 26, 2003, a 22-year-old shepherd was treated at a health post in the Popenguine District, 60 km south of Dakar, Sénégal; he reported fever, epistaxis, arthralgia, myalgia of the lower limbs, and dark urine for the past 2 days. Without biologic confirmation of the infection, he was treated for malaria with two intravenous injections of quinine, followed by oral administration of chloroquine.

On January 31, the patient had a temperature of 39°C, conjunctival jaundice, bleeding gums, and was vomiting blood. He was seen again at the health post and was given antimicrobial drugs, intravenous quinine, and vitamin K; the next day, the bleeding stopped and the fever subsided. A serum sample was sent to the World Health Organization Collaborative Centre for Arboviruses and Viral Hemorrhagic Fevers at the Institut Pasteur, Dakar. Tests for anti-CCHF specific immunoglobulin (Ig) M antibody by enzyme-linked immunosorbent assay (ELISA) were positive, and CCHF virus by isolation on cell cultures (AP61 and Vero cells) and reverse transcriptase-polymerase chain reaction (RT-PCR) were negative. From January 31 to February 10, the IgM titer increased from 1/3,200 to >1/12,800 and IgG titer increased from 1/200 to 1/6,400.

Examination of the patient on February 10 showed he had recovered without sequelae, and no trace of tick bites was found. The patient stated that he had not traveled, noticed any tick bites, slaughtered any animals, or

been in contact with people with fever for several weeks before his illness. He lived in close proximity to goats and cattle, but no blood samples were taken from these animals. Although no ticks were found on nearby goats, 10 *Amblyomma* and *Hyalomma* ticks were collected from three cattle. Ticks were negative for CCHF virus isolation on suckling mice and RT-PCR amplification.

No other case of fever accompanied by hemorrhage was reported in the area, and none of the patient's 14 close contacts became ill. Of the four close contacts from whom blood samples were taken, analyses for IgM and IgG antibodies against CCHF virus were negative by ELISA.

While no clinical case of CCHF has ever been reported in Senegal, studies dating from 1969 indicate that CCHF virus had been found in various locations in the country (2,3). In the village of Bandia, in the same district where the reported case was observed, a study conducted from 1986 to 1988 showed a prevalence of anti-CCHF IgG of 3.2% in the human population (4). Another study, conducted in the same area from 1989 to 1992, showed seroconversions for several ruminants and isolated the virus from ticks (5).

During CCHF outbreaks, an average of 30% of people who had the disease died (case-fatality ratio). It is often discovered during nosocomial outbreaks, as was the case in Mauritania, a country on Sénégal's northern border, in 2003 (P. Nabeth, unpub. data). To prevent outbreaks of CCHF, public awareness campaigns aimed at the populations most at risk—livestock farmers, butchers, and health personnel—must be conducted, and the epidemiologic alert systems must be strengthened. In addition, conditions that enhance maintenance of the virus in nature and its transmission to humans must be better understood so adequate control measures can be developed.

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Influenza among U.K. Pilgrims to Hajj, 2003

To the Editor: Each year, approximately 2 million Muslims travel from all over the world to participate in hajj. Approximately 22,000 pilgrims travel from the United Kingdom to Makkah, Saudi Arabia; of those, approximately 1,000 person reside in the east end of London. In the past, infectious diseases research conducted during these pilgrimages focused on meningococcal disease because of outbreaks associated with the hajj. Since 2000, the dates of the hajj have been moved back into the winter season; this time change could lead to a seasonal increase in outbreaks of respiratory infections caused by influenza and other viruses. From 1991 to 1992, influenza A was a common cause of respiratory infection in pilgrims tested in Makkah (1). However, the incidence rate of influenza among pilgrims from Europe is not well-known. A previous study of influenza-like illness among pilgrims from Pakistan reported rates of 36% in influenza-vaccinated pilgrims and 62% in influenza-nonvaccinated pilgrims; these results were based on clinical endpoints without microbiologic confirmation (2).

We assessed the risk for influenza infection among a cohort of pilgrims from the east end of London who participated in the hajj in 2003. From December 2002 to January 2003, we enrolled 115 participants who planned to take part in hajj in 2003. The study was approved by the North London Multicentre Research Ethics Committee and the Trustees of East London Mosque. Informed consent was obtained through appropriate translators. All participants attended the East London Mosque, Whitechapel, London; 30 were vaccinated with influenza vaccine (A/New Caledonia/20/99 [H1N1]-like strain, A /Moscow/10/99 [H3N2]-like strain, B/Sichuan/379/99-like strain). Venous blood samples were collected, and questionnaires were completed before the participants departed for the hajj and within 2–3 weeks of their return in February to March 2003.

Tests for influenza A and B were conducted by using hemagglutination

Table. Seroconversion and respiratory symptoms due to influenza infection and vaccination status among U.K. pilgrims

Influenza vaccination in autumn 2002	Seroconversion		Respiratory symptoms	
	Yes	No	Yes	No
Vaccinated	9	21	23	7
Nonvaccinated	35	50	70	15
Total	44	71	93	22

inhibition against the following influenza antigens: A/NewCalidonia/20/99, A/Wuhan/371/91, A/Sydney/5/97, A/Panama/2007/99, B/Sichuan/379/99, and B/Harbin/7/94. A diagnosis of influenza was made based on seroconversion with at least a fourfold rise in antibody titer. Based on seroconversion, the influenza attack rate among all pilgrims was 38% (44/115). The attack rate was 30% among the vaccinated and 41% among the nonvaccinated participants (Table) (odds ratio for influenza in vaccinees = 0.61, p = 0.28). Of the 44 patients, 42 (37%) were infected with influenza A H3N2; 1 had influenza A H1N1, and 1 had influenza B infection. Six influenza A H3N2 patients were dually infected; two patients seroconverted to A H1N1, and four patients seroconverted to influenza B. Nearly half (21/44) of the patients with influenza received a course of antimicrobial drugs while on the hajj compared with 38% (27/71) of those who did not seroconvert. The attack rate in the vaccinated patients was lower than the rate in nonvaccinated patients, which is consistent with some protective effect of the influenza vaccine.

Even though blood was collected from five convalescing patients within 3 weeks of their return from the hajj, some of the patients may have acquired influenza B infection immediately after their return to the United Kingdom, as it was the main strain circulating in the United Kingdom in late February to March 2003. Many pilgrims from throughout the world, some of whom may carry H3N2 drift variants, mingle closely during the hajj. This type of exposure increases the risk for worldwide spread of new drift variants and other contagious respiratory diseases (3). Given the potential for the high influenza attack rate documented in this study, all pilgrims, regardless of age, should be offered influenza vaccination before they travel on the hajj during winter months. On-site testing for influenza should be available to medical services in Makkah (and countries of origin), and treatment with a neuraminidase inhibitor should be offered to persons who test positive and have been symptomatic for <48 hours (4). This treatment should lessen the transmission risk to pilgrims during the crowded events during travel and on their return home (5). When pilgrims return from the hajj, physicians should be informed that pilgrims may bring back new drift variants of influenza; physicians should consider the diagnosis and treat persons at risk and their close contacts (4).

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Streptomyces thermovulgaris Bacteremia in Crohn's Disease Patient

To the Editor: Invasive infections with *Streptomyces* spp. are rare; in reference to two cases reported in Emerging Infectious Diseases (1,2), we describe here the first documented case of bacteremia with *Streptomyces thermovulgaris*. An 81-year-old woman was admitted to the emergency room of Diakonessenhuis, Utrecht, the Netherlands, with severe abdominal pain in the right lower quadrant and feculent vomitus. The patient had a history of Crohn's disease, for which she had undergone resection of the ileum and cecum, and was receiving high-dose corticosteroid therapy (prednisone 25 mg

daily). The patient also had steroid-induced osteoporosis and an internal pacemaker. On admission, the patient had a temperature of 35.6°C, a leukocyte count of 8.4 x 10^9/L with 30% bandforms, and a C-reactive protein level of 18 mg/L. Moreover, the patient had severe metabolic acidosis and was hemodynamically unstable, suggesting septic shock subsequent to a presumed bowel perforation.

Blood samples were drawn and cultured, and empiric treatment was initiated with ceftriaxone and metronidazole. Exploratory laparotomy showed colonic inflammation. The patient was transferred to the intensive care unit postoperatively. On day 9 of hospitalization, the blood culture taken on day 1 before antimicrobial drug was started, turned positive in the automatic blood culture system. Gram staining showed gram-positive nocardioform rods, and subculture on tryptic soy agar with 7% sheep blood yielded polymorphous colonies that sunk into the agar and showed filamentous growth.

Despite intensive antimicrobial and supportive therapy, sepsis progressed into multiple organ failure with severe neuropathy. On day 25 of hospitalization, the patient died. Autopsy demonstrated no possible infective focus except severe inflammation of the colon and distal ileum. Permission for cerebral section was not granted. The blood isolate showed strictly aerobic growth at 37°C and 50°C with β-hemolysis and was positive for catalase, caseinase, gelatinase, and nitrate reduction; the isolate was negative for oxidase, urease, and esculin hydrolysis and was nonmotile at 37°C. The isolate was sent to the National Institute of Public Health, Bilthoven, the Netherlands, for identification. Biochemical analysis, fatty acid analysis, and 16S rRNA typing identified the strain as *S. thermovulgaris*. The strain was susceptible to ceftriaxone (MIC 0.32 μg/mL) by Etest (BA Biodisk, Solna, Sweden). Further susceptibility testing performed by agar diffusion showed that the organism was also susceptible to amoxicillin, vancomycin, a combination of trimethoprim and sulfamethoxazole, and erythromycin.

Bacteremia produced by a strain of *S. thermovulgaris*, as we report here, is the first documented case of isolation of this microorganism from human material. The streptomycetes are classified as a separate genus within the aerobic actinomycetes and are most well known for the many antimicrobial substances isolated from the approximately 600 different species (3). Streptomycetes, aerobic, spore-forming, gram-positive bacteria with filamentous growth, are ubiquitous in soil and can cause mycetomas (4). Streptomycetes are widely distributed in terrestrial and aquatic habitats with soil, fodder, and compost as their primary reservoirs. The amount of actinomycetes (the taxonomic group to which the streptomycetes belong) in soil is estimated to be 10^7–10^8 microorganisms per gram (5), and in total biomass equal to that of all other bacteria together and slightly less than that of fungi. *S. thermovulgaris* belongs to the thermophilic streptomycetes, which do not grow at temperatures <37°C and are believed to affect biodegradation of organic waste products at higher temperatures (6). The *S. thermovulgaris* strains in the American Type Culture Collection have been isolated from manure, manured soil, and compost.

S. anulatus, *S. somaliensis*, and *S. paraguayensis* are the species that have been implicated most frequently as causing human disease, but streptomycetes such as *S. albus* (7), *S. coelicolor*, *S. lavendulae*, *S. rimosus*, *S. bikiniensis*, and *S. violaceoruber* have also been implicated (2). Invasive infections caused by streptomycetes are very rare, and few cases of bacteremia have been reported (1,2,8).

Reported clinical isolates are often associated with decreased immunity, as in the case of *S. bikiniensis* from a patient with osteosarcoma (2), and in the case of *Streptomyces* spp. from patients with HIV (9). Our patient had severe immunosuppression as a result of intensive steroid treatment for therapy-resistant Crohn's disease. Autopsy identified no possible focus of infection other than the patient's intestines, which were severely inflamed with massive ulceration. Cultures taken at autopsy were negative.

Because of the large numbers of streptomycetes in the agricultural environment, eating contaminated food probably occurs frequently; however, in healthy people it will not lead to invasive infection. In our patient, the heavily inflamed, ulcerated gut likely enhanced the opportunity for infection from the intestines.

Because it is a soil bacterium, *Streptomyces* spp. would not likely contaminate a hospital environment. Moreover, studies have never shown *Streptomyces* spp. as contaminants (10); therefore, any clinical isolate should be considered potentially relevant. After antibiotic therapy was initiated, repeated blood cultures showed no persisting *Streptomyces* bacteremia, which can be explained by the strain's susceptibility to the antimicrobial agents that were given.

In conclusion, we have described the first documented case of *S. thermovulgaris* bacteremia. Immunocompromised patients are susceptible to colonization and infection with a broad range of both common and uncommon pathogens. Although the clinical value of positive blood cultures with less common pathogens such as streptomycetes must always be carefully weighed, they may not simply be discarded as contaminants.

The work described in this article was conducted at the Diakonessenhuis Utrecht, Utrecht, the Netherlands.

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Human West Nile Virus, France

To the Editor: West Nile virus (WNV) is a mosquito-transmitted flavivirus, widely distributed in Africa, the Middle East, Asia, and southern Europe. Since the 1990s, its geographic distribution has expanded and caused epidemics of meningoencephalitis (1). Recently introduced into the United States, it expanded rapidly from New York throughout the country and caused illness in 9,862 human patients in 2003 (2). In France, the first reported WNV outbreak that affected horses and humans occurred during the summer of 1962 in the Camargue region (1). After 1965, no human or equine WNV infections were reported until September 2000, when a large outbreak of equine encephalitis occurred in France (3). No human cases were reported at that time. In September 2003, a human living in Fréjus (Département du Var, southeastern France) was diagnosed with acute WNV infection in Nice University Hospital. At the same time, an equine case was diagnosed 20 km from the patient's home; consequently, public health authorities initiated a retrospective study of patients hospitalized in the French Mediterranean region in which viral meningoencephalitis was suspected. We report four human cases from Fréjus Hospital.

Twenty patients who had been hospitalized at some time from August 1 to October 15, 2003, for febrile meningitis, encephalitis, or polyradiculoneuritis were screened. Four patients in whom cerebrospinal fluid (CSF) analysis indicated a viral cause were included. In addition, serum samples from two patients who had experienced flulike symptoms with exanthema during the same period were tested further. Serologic diagnosis of acute WNV infection was based on immunoglobulin (Ig) M-capture and direct IgG enzyme-linked immunosorbent assay followed by 80% plaque reduction neutralization titer ($PRNT_{80}$) by using the France 2000 WNV strain (3).

Patient 1, 46 years old, and patient 2, 25 years old, had a flulike syndrome with maculopapular exanthema; WNV seroconversion was seen on a pair of sera collected on days 3 and 16 for patient 1, and days 3 and 12 for patient 2, after onset of fever. Patients 3 and 4 had meningoencephalitis with maculopapular exanthema. In patient 3, a fourfold increase in WNV neutralizing antibodies was seen in serum samples on 2 consecutive days (days 3 and 15 after onset of fever). In patient 4, WNV IgM antibodies were detected in CSF (day 4 after onset of fever), and neutralizing antibodies (titer = 160) were reported in a serum specimen on day 75. Attempts to detect WNV RNA by reverse transcription–polymerase chain reaction, or to isolate the virus from serum specimens in patients 1 and 2 and CSF in patient 4, were negative because of the low level and short duration of WNV viremia (4). All patients recovered.

On the basis of serologic results, we describe the first human clinical WNV infections in France since 1964 (5). The four patients lived in the same city, had not traveled, and had an onset of their illness during the last week of August 2003. Of note, four clinical infections were identified, but many more WNV subclinical and asymptomatic infections likely occurred simultaneously.

After the reemergence of WNV in horses in the Camargue region in 2000, surveillance on sentinel birds (ducks and chickens) showed a low circulation of WNV in 2001 and 2002 in this area. Meanwhile, no clinical human or equine cases were detected. During the summer of 2003, WNV reemerged in humans 200 km east of Camargue, in the Département du Var, along the Mediterranean coast. A

study conducted on French blood donors from September to November 2000 showed low titers of WNV neutralizing antibodies in two donors originating from the Département du Var (6). However, to date, no clinical human cases have been reported in this area.

WNV must be considered as a causative agent of meningitis, encephalitis, and polyradiculoneuritis during summer and early fall in southern France. Given the capacity of WNV to cause large outbreaks, the surveillance will be extended to the entire Mediterranean coastal area.

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SARS in Teaching Hospital, Taiwan

To the Editor: During the global epidemic of severe acute respiratory syndrome (SARS), the illness was transmitted rapidly within hospitals, which created pools of persons who became infected and through whom the disease was spread. Intrahospital transmission amplified regional outbreaks and augmented spread of the illness into the community (1–3). Healthcare workers, hospital patients, and hospital visitors accounted for 18%–58% of all cases of SARS in the five countries with the largest outbreaks (1,2). The concentration of SARS among hospital staff strained hospital facilities, personnel, and finances.

National Taiwan University Hospital, established more than 100 years ago, is the first teaching hospital and the best resource in Taiwan for managing patients with illnesses that are difficult to treat. The hospital has 2,400 beds and provides primary and tertiary care services in Taipei. Taipei City was among the hardest hit areas by SARS in the world (3). From March 10 to July 23, 2003, the hospital reported 270 patients with SARS, many of whom were severely ill. The hospital treated 180 of the 665 patients with SARS reported in Taiwan, even though it was staffed by 4,450 of the country's 178,000 healthcare workers. SARS had an impact on this hospital for three likely reasons. First, the hospital identified and treated the first SARS patients in Taiwan (4,5). Second, it provided easy access through the emergency room and outpatient clinics. Febrile persons with a travel history to SARS-affected areas or other risk of SARS exposure came directly to this hospital for care. Third, many hospitals, particularly private facilities, were reluctant to report and admit patients with SARS during the early stage of the epidemic because of financial considerations and fear.

The hospital felt the brunt of the epidemic in Taiwan during early May 2003, which paralleled the severity of the SARS epidemic in Taipei (3,4,6). The maximal number of SARS patients admitted to the hospital within 24 hours was 12 on May 3. The maximal number of SARS patients reported within 24 hours was 15 on May 6; 8 patients were transferred to other hospitals on May 7. However, 18 patients stayed overnight in the emergency room on May 7. Subsequently, SARS developed in 12 emergency room healthcare workers (6,7).

Our preliminary studies showed that the average inpatient cost for patients with SARS was not higher than for patients with pneumonia, after adjustment for age, sex, and length of stay (MF Chen, unpub. data). However, SARS caused financial and operational disruptions in the hospital. During this period, hospital utilization rates decreased. Compared with the previous year's rates, outpatient and emergency visits fell to 37%, inpatient admissions fell to 29%, and surgical procedures fell to 15%. Bed occupancy decreased from 86% in May 2002 to 38% in May 2003.

SARS also imposed physical and psychological concerns on the healthcare workers.

During the later stage of the SARS epidemic, the Taiwan government offered special financial assistance to hospitals and healthcare workers as an incentive to help fight SARS. The country's National Health Insurance program compensated hospitals for the decrease in revenues, based on the hospital's reimbursement amount before the SARS epidemic. This measure was effective in motivating other hospitals to accept patients with SARS. The proportion of inpatients with SARS at the hospital dropped from 79.5% during March 10 to April 23, to 46.2% during April 24 to May 1, to 11.6% during May 2 to July 23. This financial assistance program remarkably reduced the impact on the hospital as other hospitals began treating patients with SARS.

Preparations for a medical emergency must address the availability and quality of medical care as well as the implications for public health policy, including political, legal, social, financial, and ethical issues (1). The importance of a sound financial policy cannot be overemphasized. Since the 1980s, healthcare systems have become free market enterprises. Laws and regulations are needed to allow governments to mobilize the resources of all hospitals and compensate them during health crises. Government agencies need to work together with the healthcare system, including health insurance systems and social services, well in advance of epidemic emergencies to maximize limited resources and distribute them equitably.

Democratic societies must preserve human rights (including the right to medical care and freedom from fear), while respecting and protecting the rights and safety of hospitals and healthcare workers. We now face the potential resurgence of SARS, other emerging and reemerging infectious diseases, and the threat of bioterrorism. Careful consideration of the financial issues of hospital management should be an important part of social policy. The emergence of SARS provides a reminder of the potential threat to the entire healthcare system when a new disease suddenly appears. A major lesson from the SARS experience is that government planning and intervention are required.

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Boiling and *Bacillus* Spores

To the Editor: Public health authorities rely upon "boil water" advisories to alert consumers if a potable water supply is deemed unsuitable for consumption. Holding water at a rolling boil for 1 minute will inactivate waterborne pathogens, including encysted protozoa (1–3). Spores of *Bacillus anthracis*, the agent that causes anthrax, are one of the microorganisms most refractory to inactivation by the boiling water method. This study was conducted to determine the resistance of spores of *B. anthracis* Sterne and three other strains of *Bacillus* spp. in boiling water.

B. anthracis Sterne (Colorado Serum Co., Denver, CO) was grown on soil extract peptone beef extract medium (4). Spores were harvested from the agar plates and washed four times by centrifugation with sterile distilled water, treated with 50% (vol/vol) ethanol while being shaken at 100 rpm for 2 h, then washed an additional four times by centrifugation with sterile distilled water. Spores of one of the *B. cereus* strains were obtained from a commercial source (Raven Biological Laboratories, Omaha, NE). Spores were produced in broth cultures for the other *Bacillus*

spp. The second *B. cereus* (ATCC 9592) was grown in a generic sporulation medium (5), and *B. thuringiensis* var. *israelensis* (ATCC 35646) was grown in Schaefer's medium (6). Spores were purified by gradient separation using RenoCal-76 (Bracco Diagnostics, Princeton, NJ) (6). Spore preparations were stored in 40% (vol/vol) ethanol at 5°C until used.

Duplicate experiments for each species were conducted in 1-L glass beakers containing 500 mL of municipal drinking water (21±2°C, pH 8.2±0.5, free available chlorine 0.5±0.3 mg/L). The beakers were left uncovered or covered with a watch glass. Steam was allowed to escape from the covered beakers through the mouth of the pouring spout. Water samples were injected with the spore preparations, heated to boiling on a hot plate, and held at boiling temperature for various times. Measuring the boiling times began when the sample reached a rolling boil. A thermocouple thermometer (Cole-Parmer, Vernon Hills, IL) directly above the liquid-air interface determined the air temperature above the boiling water after 5 min of exposure. At the conclusion of the various boiling times, the samples were removed from the heat source and allowed to cool at room temperature before analysis. These samples contained <0.2 mg/L of free available chlorine. Decimal dilutions of the water samples were analyzed in triplicate by the membrane filter procedure with nutrient agar (7).

Spores of all strains of the *Bacillus* spp. analyzed in this study were inactivated after boiling for 3–5 min in a covered vessel (Table). Spores still survived after 5 min of boiling in an open vessel for all of the *Bacillus* spp. Temperatures immediately above the surface of the boiling water in the covered vessels averaged 98.9°C, while the temperature immediately above the water level in the uncovered vessels averaged 77.3°C.

In a comprehensive literature review citing published reports dating back to 1882, Murray (8) noted that boiling times reported to destroy *B. anthracis* spores varied over a range of 1 to 12 min. In his own study of 17 strains of *B. anthracis*, Murray (8) found that boiling times of 5 to 10 min were required to achieve inactivation. Stein and Rogers (9) reported that vigorous boiling for 3 to 5 min destroyed spores from 43 strains of *B. anthracis*.

In our study, boiling water in a covered vessel for 3 to 5 min destroyed spores of the *Bacillus* spp. by greater than four orders of magnitude. Boiling for 5 min in an uncovered vessel was not as effective as boiling in a covered vessel and allowed all *Bacillus* spp. spores to survive. On the basis of the initial levels of spores used in this study, holding water at a rolling boil for 1–3 min in an open container would not inactivate the spores. Boiling time refers to the total time the water is held at a rolling boil and should not be confused with the first sign of bubbles from dissolved gases in the water. Since water boils at lower temperatures at higher altitudes (approximately 90°C at 3 km), boiling times must also compensate for decreased atmospheric pressure conditions (1,2).

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Table. Inactivation of *Bacillus* spp. by boiling in tap water

	Initial $\log_{10}$ CFU/mL		Boiling times[a] $\log_{10}$ CFU/mL					
			1 Min		3 Min		5 Min	
Organism	Covered	Uncovered	Covered	Uncovered	Covered	Uncovered	Covered	Uncovered
B. anthracis Sterne	4.95	4.92	0.11	ND[b]	<0[c]	2.13	<0[c]	2.01
B. cereus (commercial)	4.62	4.59	0.81	1.94	<0[c]	1.50	<0[c]	1.46
B. cereus, ATCC 9592	4.54	4.76	<0[c]	0.78	<0[c]	0.60	<0[c]	0.48
B. thuringiensis ATCC 35646	4.63	4.46	<0[c]	1.76	<0[c]	1.58	<0[c]	1.47

[a]Values are means of duplicate experiments ≤0.25 log units.
[b]ND, not determined.
[c]<0, a number reading below the detection level.

In Memoriam

Photo by F. Jacquerioz

Aniru Conteh
1942–2004

On the Front Lines of Lassa Fever

Aniru Conteh spent 25 years in his native Sierra Leone dedicated to treating patients with Lassa fever, a rodentborne viral disease, to which he ultimately succumbed on April 4, 2004. Dr. Conteh's life is a model of the dedicated healthcare worker. His colleagues hope that his death can galvanize support for healthcare workers and scientists working on the front lines with Lassa fever virus and other emerging pathogens.

The son of the local chief, Aniru Sahib Sahib Conteh was born in the small village of Jawi Folu in Eastern Province, Sierra Leone, in 1942. When Conteh was 16 years of age, his mother died, and he left school to help support the family in the capitol, Freetown. He eventually returned to school, where he studied chemistry and biology, and earned his bachelor's degree from Durham University, Freetown. After working briefly as a teacher, he enrolled in medical school at the University of Ibadan, Nigeria. He graduated in 1974 and stayed on to work at Ibadan Teaching Hospital. In 1979, Dr. Conteh returned to Sierra Leone, beginning what would be a 25-year career dedicated principally to the fight against Lassa fever.

Lassa fever was first recognized in 1969 after three nurses working at a mission hospital in North-Eastern State, Nigeria, came down with a mysterious illness (1,2). A new virus was subsequently isolated from a blood specimen sent to the Yale Arbovirus Research Unit and named Lassa after the village of origin of the first case-patient (3). A larger outbreak, 28 cases with at least 14 deaths, occurred in the same region in 1970 (4). Lassa fever was first identified in Sierra Leone in 1972 in a series of nosocomial outbreaks (5,6). The disease was found to be common in the community as well, constituting a major cause of illness and death in eastern Sierra Leone, which prompted the Centers for Disease Control (CDC) to establish a Lassa fever research and control program in Sierra Leone in 1976 (7).

Dr. Conteh willingly plunged into this hotbed of Lassa fever in 1979 when he took a post at Nixon Methodist Hospital in the eastern town of Segbwema, the central hospital of CDC's program. He was named Nixon Hospital's medical superintendent in 1980 and later served as the clinical director of the Lassa fever treatment ward . When civil war broke out in 1991, the treatment ward was moved to the relative safety of nearby Kenema Government Hospital, and Dr. Conteh continued as its director. The war eventually forced the CDC program to close, but Dr. Conteh and the Lassa fever ward carried on through the support of the British medical relief agency, Merlin.

From 1979 to 2004, Dr. Conteh treated thousands of patients with Lassa fever, becoming the unparalleled world's expert on the management of the disease, as well as contributing to research on the subject (8). He persevered despite many risks—outbreaks of Lassa fever, rebel invasions, and government counterattacks. Through various projects and experts, war and peace, and waves of refugees, Dr. Conteh stayed, continuing to treat patients in his characteristically calm and modest manner. His dedication, skill, and courage were some of the few constants in the unstable and often dangerous world around him.

In March 2004, Dr. Conteh admitted a young, pregnant woman to the Lassa ward with a presumptive diagnosis of severe Lassa fever. The patient was a volunteer nurse on the hospital's pediatric service. On March 17, after numerous unsuccessful attempts by staff members to obtain blood from the patient's arm, Dr. Conteh attempted femoral venipuncture and sustained a needlestick injury in the process. The patient died the next day. On March 23, fever developed in Dr. Conteh. Despite the administration of intravenous ribavirin, profuse vomiting and diarrhea developed a few days later; these led to hypovolemic shock and cardiac arrest, which necessitated resuscitation. Bleeding and renal failure ensued. Consultations were sought and received from medical experts around the world. The diagnosis of Lassa fever was confirmed from specimens sent to the National Institute for Communicable Diseases in South Africa. On April 4, in the cruelest irony, Aniru Conteh died of a virus that he had been combating as a physician for most of his life, a patient in a ward that he had been instrumental in establishing and maintaining.

Dr. Conteh's death represents more than a personal loss. His absence severely undermines the ability to combat Lassa fever, which remains a serious threat. Research over the years has shown that Lassa fever is endemic in Liberia, Guinea, Nigeria, and Senegal as well, and Lassa virus is now believed to infect tens of thousands of people and cause thousands of deaths yearly across West Africa (7,9–13).

What lessons can we learn? What response can we have to this tragedy? We have made progress in our global response to emerging pathogens, but many challenges remain. Perhaps the

most important response is to offer a sound base of support for combating emerging diseases where they start, relying less on the rapid influx of international experts and the long-distance shipping of specimens and more on "home grown" talent, equipped with the tools and training that they need. Achieving these goals will be difficult. Beyond supplying medicine and laboratory equipment, tackling the problem will require addressing such complex issues as low salaries and "brain drain," civil unrest, corruption, and human rights. Dr. Conteh was exceptional because he persisted in the face of these challenges, but we cannot routinely depend on such heroes. Governments in developing countries, with international support, need to build the base to create stable job and training opportunities, adequate physical infrastructure, and safe working environments to foster the development of local expertise and encourage local physicians and scientists to help fill the role vacated by Dr. Conteh. Ultimately, containing emerging diseases depends on the Aniru Contehs of the world. The more support we provide to people on the front lines, the healthier and safer we all will be.

Aniru Conteh is survived by his wife, Sarah, three sons, and two daughters.

Acknowledgments

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Correction, vol. 10, no. 9

In "SARS-CoV Antibody Prevalence in All Hong Kong Patient Contacts" by Gabriel M. Leung et al., errors occurred on p. 1654. The seventh sentence read "those who declined testing" but should have been "those who consented to testing."

The corrected sentence reads as follows: However, those who consented to testing were more likely to report more frequent contact and closer relationships with SARS patients, more febrile or respiratory illness episodes since February, and a travel history to SARS-affected regions, which may have biased our seroprevalence estimate upwards.

The corrected article appears online at http://www.cdc.gov/ncidod/EID/vol10no9/02-0155.htm

We regret any confusion these errors may have caused.

Conference Summary

New and Re-Emerging Infectious Diseases

The seventh annual Conference on New and Re-Emerging Infectious Diseases, April 15–16, 2004, was hosted by the Center for Zoonoses Research and the College of Veterinary Medicine at the University of Illinois at Urbana-Champaign. The conference featured eight speakers and 31 poster presentations.

The conference was opened with a presentation on *Yersinia enterocolitica*, a gram-negative enteric human pathogen that causes enterocolitis. Invasin is the primary requirement for efficient translocation of the bacteria across the intestinal epithelium; the identification of both positive and negative regulators of its expression have been identified.

The emergence of *Mycoplasma gallisepticum*, which causes severe conjunctivitis in house finches, was described. The strain of *M. gallisepticum* that affects house finches, a new host recently introduced to North America, is a novel strain that emerged recently.

Current therapies for microsporidiosis, a serious opportunistic infection in persons with AIDS, organ transplant recipients, children, travelers, contact lens wearers, and the elderly, were reviewed.

Studies on the human fungal pathogen *Cryptococcus neoformans*, which causes life-threatening infections of the central nervous system, most commonly in immunocompromised hosts, were discussed. The studies focused on signaling cascades that govern virulence and an unusual mating type locus linked to differentiation and virulence.

Release of the variant surface antigens of African trypanosomes, agents of a reemerging infectious disease in sub-Saharan Africa, occurs not only by proteolysis but also by glycosylphosphatidylinositol-anchor hydrolysis through a phospholipase present in the parasite surface.

To characterize the human colonic response to *Shigella* and *Entamoeba histolytica* at the molecular level, differential transcription of nearly 40,000 human genes in sections of human colonic xenografts that had been infected with *Shigella flexneri* or *E. histolytica* was measured. The results indicated increased expression of genes encoding proteins involved in stress, hypoxic responses, immune and inflammatory responses, responses to tissue injury and tissue repair, cytokines, and chemokines.

Studies on sortases, membrane-anchored transpeptidases that cleave surface proteins, were described. Because sortases of *Staphylococcus aureus* are required for animal infections, inhibitors that disrupt the activity of sortases may be therapeutically useful.

The conference was concluded with a description of the severe acute respiratory syndrome (SARS) epidemic in China. China's handling of the epidemic has been critiqued by the international community, but a balanced view of events has shown that many measures undertaken by officials, such as large-scale quarantines, mandatory fever-screening checkpoints, arrival and departure monitoring at airports, populationwide surveillance, community infection control, and designating SARS-only hospitals, worked effectively to contain this infectious disease.

The proceedings are available in PDF format at http://www.cvm.unic.edu/czr/

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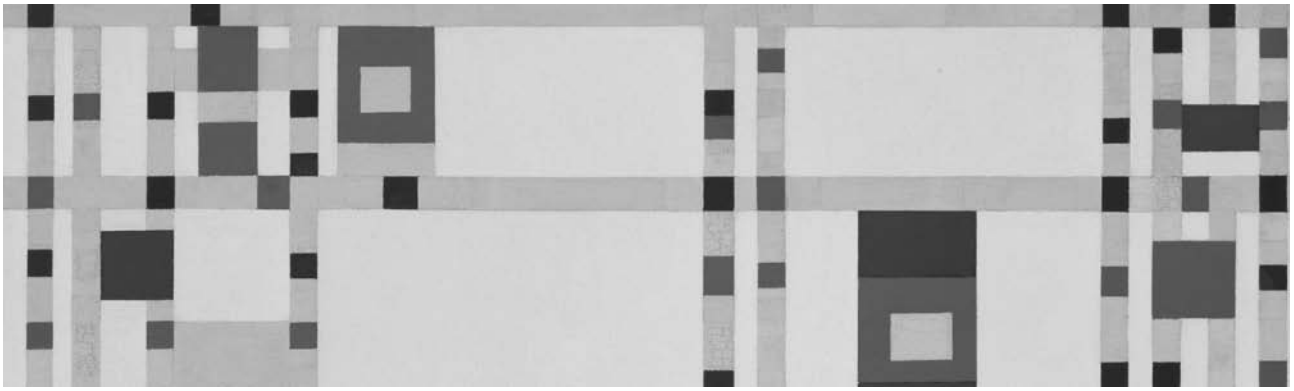


Piet Mondrian (1872–1944). Broadway Boogie Woogie (1942–1943). Oil on canvas, 127 cm x 127 cm. The Museum of Modern Art, New York, NY, USA
Digital image: The Museum of Modern Art/Licensed by SCALA/ART Resource, NY

Molecular Techniques and the True Content of Reality

Polyxeni Potter

"Everything was spotless white, like a laboratory. In a light smock, with his clean-shaven face, taciturn, wearing heavy glasses, Mondrian seemed more a scientist or priest than an artist. The only relief to all the white were large matboards, rectangles in yellow, red and blue, hung in asymmetric arrangements on all the walls" (1). This description of Piet Mondrian's New York studio sheds light on the man who went beyond all efforts of his generation to achieve abstraction in search of absolute reality.

Mondrian's incongruous appearance and even his name (changed from Mondriaan) reflected his transformation during an artistic career that spanned two world wars. Over the 20 years during which he studied abstraction, he steadily moved toward simplicity and purity. He abandoned all that was representational, turning himself from a painter of landscapes and flowers to one that tolerated only horizontal and vertical lines, flat surfaces, and primary colors. Forging a style that was in its essence mathematical, he selected single motifs and worked on them until they were completely stripped of form and reduced to lines or grids: "I saw the ocean as a series of pluses and minuses" (2).

Mondrian was born into a family of artists in Amersfoort, Holland, and was brought up a Calvinist. His early work, mostly landscapes of the Dutch countryside, bespoke the realism featured in his academic training and a sense of order and surface geometry reminiscent of Jan Steen. Influenced by the work of Vincent van Gogh and an interest in theosophy, his paintings became increasingly abstract (3).

Theosophy, a philosophic movement of the late 19th century that focused on the spiritual structure of the universe, also influenced the work of Wassily Kandinsky, Kazimer Malevich, and other contemporaries (4). Mondrian traveled to Paris, where he met Georges Braque and other leading artists and was exposed to the abstracting qualities of cubism and the primary colors of fauvism. His work in Paris culminated in a new art movement known as De Stijl or neoplasticism.

The term neoplasticism was coined by Mondrian's friend the Dutch mathematician and theosophist M.J.H. Schoenmaekers. "Plastic" referred to a formal structure underlying everything in nature. In abstract art, distracting elements around this fundamental structure were removed, leaving fragments of objects or, in Mondrian's work, black bands and color rectangles. The challenge was to find, out of infinite possibilities, the right relation between these bands and the rectangles they formed.

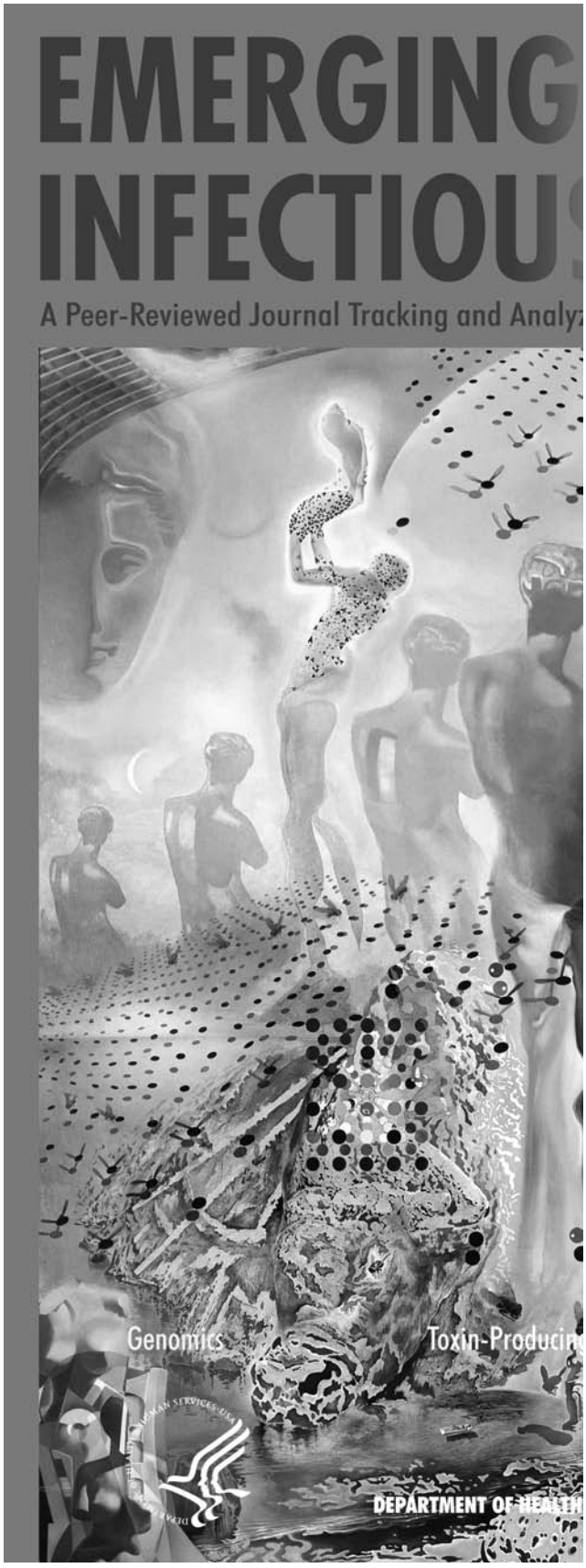
Establishing the right relation between line and color (band and rectangle) was the path to "pure reality," which Mondrian defined as equilibrium "through the balance of unequal but equivalent oppositions" (3). Not a single line or color could be moved without disrupting this balance. "The rhythm of relations of color and size," he wrote in Natural Reality and Abstract Reality, "makes the absolute appear in the relativity of time and space" (3). Mondrian's principles of rigorous abstraction, refined geometry, and exquisite nonsymmetrical balance have influenced modern architectural, industrial, and other nonfigurative design.

When Mondrian arrived in New York during World War II, he was 70 years old and in poor health, yet his creativity reached a new height before his death of pneumonia in 1944. Broadway Boogie Woogie, on this month's cover of

Emerging Infectious Diseases, was the last painting he finished. Born of sheer fascination with the vital culture of 1940s New York, this celebrated work seems to synthesize the elements of his artistic philosophy. As if finally confident in the sound structural relations between bands and rectangles of color, Mondrian made one more radical abstraction. Modifying his hallmark black grid, he integrated bands and color in a series of small, unequal but equivalent rectangles. The result seems an exuberant abstraction of New York itself, a fluorescent skeleton of its architectural blocks, the rhythm of its heartbeat, the lights of its nightlife on an infinite flickering marquis.

A lover of music and dance, Mondrian was in tune with the culture of his day. He clearly knew boogie-woogie, the dynamic, colorful music that reached its peak in 1938, when Albert Ammons, Pet Johnson, and Meade Lux Lewis brought it to Carnegie Hall (5). The repetitive eight-to-the-bar bass line of boogie-woogie blues structure found a perfect home in Mondrian's disciplined rectangles. Yellow, red, blue, gray, white boxes, aligned in regular intervals and punctuated by improvised riffs, form a seamless, perfectly balanced grid. Brimming with joyous movement and effortless rhythm, a parade of blinking steps engages the viewer in an ingenious visual dance.

The equilibrium Mondrian sensed in the universe and sought in radical abstraction is well known to biologists. Modern molecular techniques, stripping organisms of all but their genetic base, array clumped fragments—DNA fingerprints—the biologist's version of Mondrian's grid. The fragments, used to type and characterize agents such as *Pneumocystis jirovecii*, causative agent of *Pneumocystis* pneumonia, provide scientists a glimpse of pure reality, along with information on sources of infection, patterns of transmission, and potential emergence of antimicrobial resistance (6).



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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends
Vol. 10, No. 11, November 2004

Upcoming Issue

Look in the November issue for the following topics:

Human Papillomavirus Vaccination Programs

Postoperative Infections

Infections after Breast Surgery and Cesarean Section

Lymphedema Management and Histopathology

Topographic Changes in SARS Coronavirus-infected Cells

Early Diagnostic Marker of SARS

Commercial Logging and the HIV Epidemic, Africa

Decreased Susceptibility of *Haemophilus influenzae* to Levofloxacin among Children, Hong Kong

Human Infection caused by *Clostridium hathewayi*

International Conference on Emerging Infectious Diseases

International Conference on Women and Infectious Diseases

Complete list of articles in the November issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

October 27, 2004
Public Health Research Institute Symposium - Infectious Diseases: Global Threats to Public Health
Newark, NJ, USA
http://www.phri.org/news/news_events50526.asp

October 30–November 2, 2004
44th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
Washington, DC, USA
Contact: 202-737-3600 or ICAAC@asmusa.org
http://www.icaac.org

November 7–11, 2004
53rd Annual Meeting, American Society of Tropical Medicine and Hygiene
Miami, FL, USA
Contact: 847-480-9592 or astmh@astmh.org

November 9–10, 2004
Antimicrobial Resistance and Emerging Infections: A Public Health Perspective
Philadelphia, PA, USA
Contact: 617-983-6285
neoffice@nltn.org
http://www.uphs.upenn.edu/epaasm/

November 12–14, 2004
Symposium, The Changing Landscape of Vaccine Development: Translating Vaccines for Emerging Diseases and Biodefense to the Marketplace
Galveston, TX, USA
Contact: 409-747-8151 or
http://www.utmb.edu/scvd

November 17–20, 2004
5th Louis Pasteur Conference on Infectious Diseases
Paris, France
Abstract submission deadline: September 2, 2004
clp@pasteur.fr
http://www.pasteur.fr/infosci/conf/sb/CLP5

EMERGING INFECTIOUS DISEASES
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 provide 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 two. 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 two. 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 1,000–1,500 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 two); and a brief biographical sketch of first author—both authors if only two. 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 one 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 (effective January 2005). 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.