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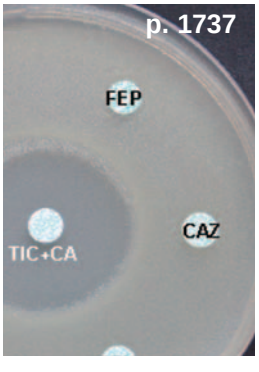

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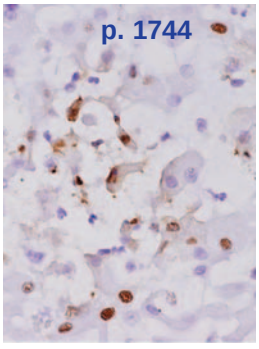
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Prophylaxis and Treatment of Pregnant Women for Emerging Infections and Bioterrorism Emergencies

Joanne Cono,* Janet D. Cragan,* Denise J. Jamieson,* and Sonja A. Rasmussen*

Emerging infectious disease outbreaks and bioterrorism attacks warrant urgent public health and medical responses. Response plans for these events may include use of medications and vaccines for which the effects on pregnant women and fetuses are unknown. Healthcare providers must be able to discuss the benefits and risks of these interventions with their pregnant patients. Recent experiences with outbreaks of severe acute respiratory syndrome, monkeypox, and anthrax, as well as response planning for bioterrorism and pandemic influenza, illustrate the challenges of making recommendations about treatment and prophylaxis for pregnant women. Understanding the physiology of pregnancy, the factors that influence the teratogenic potential of medications and vaccines, and the infection control measures that may stop an outbreak will aid planners in making recommendations for care of pregnant women during large-scale infectious disease emergencies.

A primary goal of public health response to emerging infections and bioterrorism attacks is to limit illness and death by providing the safest and most effective medical prophylaxis and treatment measures (medical countermeasures) in a timely manner to persons at greatest risk. Information on the effectiveness and safety of some medical countermeasures is limited for the general population, and even less information is available for pregnant women (*1*). Physiologic changes during pregnancy may change the safety profile and efficacy of medications and vaccines for pregnant women. The potential effect of many of these measures on the fetus is unknown. These factors could influence a clinician's willingness to prescribe and a woman's decision to accept potentially lifesaving treatments.

The circumstances under which exposure to medications or vaccines during pregnancy occurs must be taken into account. For example, when a pregnant woman has a serious acute infection, such as severe acute respiratory syndrome (SARS), anthrax, or a pandemic strain of influenza, appropriate timely treatment must be provided to preserve her health. When multiple therapeutic interventions of similar efficacy are available, consideration can be given to choosing the therapy that will best safeguard maternal health and the well-being of the embryo or fetus. In contrast, when a pregnant woman has been exposed to a serious infection but is not acutely ill, the choice of whether to provide prophylaxis or empirical treatment depends on several factors including the nature and certainty of the exposure, likelihood and potential severity of her infection, and gestational age at which exposure occurred. Inadvertent exposure to a medication or vaccine also may occur during pregnancy. An estimated half of pregnancies in the United States are unplanned (*2*); thus, a woman infected with or exposed to a serious acute infection might receive emergency prophylactic or treatment measures during the early weeks of gestation before a pregnancy is recognized. In this situation, opportunity to weigh the risks and benefits to a pregnancy before exposure to the medication or vaccine is missed; instead, consideration must focus on any effects these measures may have had on the fetus.

Special Physiologic Features of Pregnancy

Physiologic changes in maternal organ systems during pregnancy, beginning in the first trimester and peaking in the second, can have effects on the pharmacokinetics of

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some drugs. A drug's pharmacokinetics (i.e., attainment and maintenance of the appropriate drug serum concentration) are affected by 4 major factors: absorption, distribution, metabolism, and elimination (*3*). Because physiologic changes are evolving continuously during pregnancy, pharmacokinetic information must be interpreted with regard to gestational age (*4*).

Changes in the maternal gastrointestinal and cardiovascular systems affect drug absorption. Delayed gastric emptying and decreased gastrointestinal motility, largely due to elevated levels of progesterone that relax smooth muscle, influence absorption of drugs taken orally. In addition, a decrease in gastric acid secretion results in higher gastric pH, which affects absorption of weak acids and bases (*4*,*5*). Increased blood flow to the stomach and small intestine, resulting from changes in the cardiovascular system (most notably, a 30%–50% increase in cardiac output) (*4*), increases absorption of drugs taken orally (*3*). Elevated blood flow also increases the absorption of drugs administered intramuscularly. However, late in pregnancy decreased blood flow to the lower extremities may result in decreased absorption in these areas (*6*).

Plasma volume increases by 30%–50% during pregnancy to meet the increased requirements of uterine-placental circulation. This increase results in a higher volume of distribution for most drugs. As the plasma volume expands, the volumes of extracellular fluid and total body water also increase. Total body weight and body fat increase throughout pregnancy, resulting in a larger volume of distribution, particularly for fat-soluble drugs (*3*). As plasma albumin concentrations decrease, so do concentrations of proteins available for binding, resulting in higher circulating amounts of free, unbound drug (*5*). However, unbound drugs may be more easily cleared by the kidney and liver, which may offset the effect of the increased volume of distribution (*7*).

During pregnancy, enzyme activity in the liver, a major site for drug metabolism, changes considerably. Activity of certain liver cytochromes (e.g., CYP3A4, CYP2D6) is increased during pregnancy. However, activity of CYP1A2, the enzyme responsible for metabolism of approximately half of all pharmacologic agents, is decreased. Increases in estrogen and progesterone during pregnancy also alter hepatic enzyme activity (*3*,*4*).

Several factors affect drug elimination during pregnancy. Changes in kidney function parallel the changes in cardiac function, with a 60%–80% increase in renal blood flow and a 50% increase in the glomerular filtration rate. Renal secretion and reabsorption increase by ≈20% (*5*). Drug elimination also occurs through respiration, which becomes a more important route during pregnancy because of changes in pulmonary function, including increased tidal volume, minute volume, and respiratory rate (*3*).

Although these physiologic changes during pregnancy can have varied and substantial effects on drug pharmacokinetics, data about their effects are limited. No evidence-based guidelines exist for how drug dosing should be altered during pregnancy (*1*). Thus, pregnant women are usually given medication doses and schedules identical to those of nonpregnant adults, despite evidence that effective therapeutic levels and toxicity may be altered by pregnancy (*4*).

Vaccine efficacy during pregnancy is another area that merits further investigation. During pregnancy, the maternal immune system undergoes extensive changes. Although these changes are not well understood, a shift away from cell-mediated immunity and toward humoral immunity appears to occur. How these immune alterations affect maternal response to vaccination during pregnancy is unknown (*8*). However, limited data on several vaccines (e.g., hepatitis B, influenza, group B *Streptococcus*) suggest that the immune response of pregnant women to these vaccines is similar to that of nonpregnant women (*9*).

Teratogenic Potential of Medications and Vaccines

Whether use of a medication or vaccine is harmful to the embryo or fetus depends on multiple factors, including the nature of the agent (e.g., live versus killed vaccine), its dose and route of administration, timing of use during gestation, concomitant use of other agents, nature of the infection being treated or prevented, and genetic susceptibility of the pregnant woman and of the embryo or fetus. Potential adverse effects of an exposure on the embryo or fetus include spontaneous pregnancy loss, structural malformations, intrauterine growth restriction, preterm delivery, hearing loss, and neurobehavioral abnormalities, among others. Timing of exposure during gestation is particularly critical. Organogenesis, the period of organ formation, extends from 15 to 60 days after fertilization (≈4–11 weeks after the start of the last menstrual period) (*10*). Before organogenesis, harmful exposures are most likely to result in spontaneous pregnancy loss, although some embryos that survive can be adversely affected (*11*). After this time, structural abnormalities are less likely to occur, although damage to a normally formed organ is still possible (*12*). In addition, some teratogenic medications have a narrow window of exposure when their use results in malformations. For example, thalidomide is believed to produce malformations only when used 34–50 days after the beginning of the last menstrual period (*13*). In contrast, adverse outcomes such as growth retardation and functional abnormalities can result from later exposures. Angiotensin-converting enzyme inhibitors have been associated with impaired renal function in the newborn when used to treat maternal hypertension during the latter half of pregnancy (*13*).

In the United States, the reproductive effects of medications and vaccines are usually assessed in animal studies before these products are licensed for human use. Efficacy in humans is evaluated in premarketing clinical trials. However, because of ethical concerns about exposing an embryo or fetus to an agent with unknown effects, reproductive studies are not performed in humans before licensure, and pregnant women have traditionally been excluded from clinical trials of efficacy (*14*). Although animal studies can be useful in evaluating an agent's potential for adverse reproductive effects, they are not always predictive of the effects in humans.

For these reasons, information about the effects of medications and vaccines during pregnancy is usually obtained from data collected after these agents are in use in the population. These data take the form of adverse event reports, case series, prospective exposure registries, and cohort and case-control studies, each of which has its own methodologic strengths and limitations (*15*). Conclusive information can be difficult to obtain from these studies because of low levels of use of individual medications or vaccines in the population outside of an emergency setting and the difficulty of separating reproductive effects of the medication or vaccine from those of the underlying infection or other genetic and environmental factors. A 2001 review of available information about medications approved by the US Food and Drug Administration (FDA) from 1980 through 2000 concluded that insufficient information existed to assess the teratogenic potential of >90% of these drugs (*16*).

In 1979, to help healthcare providers assess potential risks and benefits of medications during pregnancy, FDA developed a use-in-pregnancy rating system (21 CFR 201.57). This system labels drugs on the basis of assessment of their relative risk to the fetus and their potential benefit to the mother (*17*). Ranging from category A through X (Table 1), this scale uses available data from animal reproductive and human studies. This rating system is used widely by clinicians in the United States, but it has several shortcomings. These include the fact that medications in the same letter category may have different magnitudes of fetal risk, most medications are rated category C (i.e., insufficient information is available to assess their potential risk and benefit during pregnancy), and the rating is not routinely updated when new information becomes available (*18*). In addition, this rating system does not address the effects of gestational timing of exposure or of physiologic changes that occur during pregnancy (*18,19*). FDA recognizes these limitations and is working to improve communication about the risks and safety of medication use during pregnancy (*20*).

Use of Medical Countermeasures in Prophylaxis and Treatment during Emerging Infection and Bioterrorism Emergencies

Limited information about the effects of medications and vaccines during pregnancy can pose a dilemma for women and healthcare providers when making decisions about their use. Pregnant women may be reluctant to receive, or healthcare providers may be reluctant to prescribe, needed medications or vaccines because of fear of harming the fetus. However, if a pregnant woman has a serious acute infection or has been exposed to a potentially life-threatening infection, treatment or prophylaxis can be lifesaving for both mother and fetus. Physicians and women often overestimate the risk to the fetus of medication use during pregnancy (*21*). As a result, needed interventions may be withheld or pregnancies perceived to be at risk may be terminated. Decisions about the treatment or prophylaxis of emerging infections must take into account the effect on the mother's health and the potential risks for the embryo or fetus.

In preparation for potential bioterrorism emergencies, the US government has stockpiled medications and vaccines, most of which are rated by FDA as 1 of the categories B through X, which indicates that they could pose a risk to the unborn fetus or that insufficient information exists to evaluate their potential fetal risk (Table 2). Some of these products (e.g., ciprofloxacin, gentamicin, and doxycycline) are commonly used in routine healthcare, but others (e.g., smallpox and anthrax vaccines) are reserved for emergency preparedness and response activities and for deployed military personnel.

Some emergency response medications and vaccines fall outside of the FDA labeling system because they are not licensed by FDA. Some are newly developed and still in prelicensure clinical trials; others are no longer licensed and predate the classification system. In these instances, the Centers for Disease Control and Prevention (CDC) holds Investigational New Drug protocols, approved by

Table 1. US Food and Drug Administration use-in-pregnancy drug classifications (21 CFR 201.57)

Category	Description
A	Well-controlled studies in humans fail to demonstrate fetal risk.
B	Human risk is relatively unlikely because of negative results in animal studies and no human studies, or positive results in animal studies and negative results in human studies.
C	Human fetal risk is unknown; positive results in animal studies (or no animal studies) and no human studies.
D	Evidence of human fetal risk; however, drug benefits may outweigh risks.
X	Positive results in animal studies or evidence of human fetal risk; use in pregnant women is contraindicated.

Table 2. US Food and Drug Administration pregnancy classifications of bioterrorism medical countermeasures

Medication	Category	Potential use
Amoxicillin	B	Anthrax
Ampicillin	B	Anthrax
Botulinum antitoxin	Unlicensed/C*	Botulism
Cidofovir	C	Vaccinia, monkeypox, smallpox
Ciprofloxacin	C	Anthrax, plague, tularemia
Doxycycline	D	Anthrax, plague, tularemia
Gentamicin	D	Plague, tularemia
Penicillin	B	Anthrax
Smallpox vaccine	Unlicensed/C*	Smallpox, monkeypox
Vaccinia immune globulin	C	Vaccinia

*Multiple products/preparations.

the FDA, which permit distribution and use of these agents in emergency situations. These protocols include extensive educational materials for potential recipients about the risks and benefits of treatment and include special considerations for pregnant women.

Although limiting fetal exposure to treatments that may pose unknown risks is optimal, protecting the life of the mother is key in protecting the fetus. In an emergency setting with a high risk for life-threatening exposure to an infectious pathogen, recommendations likely will call for the use of vaccination and prophylactic medications, when they are available, for pregnant women, despite unknown risks to the fetus. Other measures that can protect persons who are unable or choose not to receive vaccination or prophylactic medications include limiting exposure to persons who may be infectious, avoiding public gatherings, and restricting travel to affected areas.

Issues in Treatment and Prophylaxis of Emerging Infections and Bioterrorism Attacks

In recent years, the public health and medical communities have faced several emerging infectious disease outbreaks, including SARS and monkeypox, and much consideration has been given to preparation for a future influenza pandemic. In addition, experience with bioterrorism attacks (anthrax) and emergency response preparedness (smallpox vaccination) has been gained. These events required careful consideration of recommendations for the care of pregnant women.

The SARS outbreak of 2003, caused by a newly identified coronavirus, affected >8,000 persons worldwide (*22*). Reports suggest that the clinical course and outcomes of SARS might be more severe for pregnant than for nonpregnant women (*23*). Identifying appropriate treatment modalities during the SARS outbreak was challenging, given the lack of information about the newly identified disease. Ribavirin was initially chosen because of its broad antiviral spectrum. Corticosteroids were used in an attempt to limit the tissue damage caused by the inflammatory response (*24*). However, issues regarding the teratogenicity of these medications have been raised, further complicating decisions about their use during pregnancy. Some animal studies have suggested that ribavirin is teratogenic, but limited experience is available regarding its effects on human pregnancies (*25*). Animal studies and some human studies have demonstrated an increased risk for birth defects when corticosteroids are used during pregnancy (*26*). In spite of this information, all but 1 of the 12 pregnant women with SARS reported from Hong Kong Special Administrative Region, People's Republic of China Special Administrative Region, People's Republic of China received ribavirin and corticosteroid treatment (*22*), probably because their illness was life-threatening. On the basis of more recent data, the efficacy of ribavirin and corticosteroids in the treatment of patients with SARS has been questioned (*24*). Other medications, such as interferons, have been proposed for use in future SARS outbreaks, but use of these medications in pregnant women may also be of concern.

In June 2003, the first outbreak of monkeypox in the Western Hemisphere occurred in the United States (*27*). Because of the high death rate associated with monkeypox on the African continent (*28*) and lack of experience with monkeypox in the United States, CDC recommended smallpox (vaccinia) vaccination (≈85% effective against monkeypox) (*29*). The outbreak was traced to importation of infected rodents that infected pet prairie dogs and other small mammals kept as pets. Smallpox vaccination during pregnancy poses a low risk for fetal vaccinia, which can lead to preterm birth, and fetal and neonatal death (*30*,*31*). However, women who were exposed were advised to receive the smallpox vaccine regardless of their pregnancy status (*32*), given the life-threatening risk associated with monkeypox infection.

Planning for a future influenza pandemic must include specific considerations for pregnant women (*33*). Because pregnancy has been shown to increase the risk for influenza-associated complications (*34*), pregnant women are considered a high-risk group and are recommended to receive influenza vaccination during interpandemic years (*35*). This vaccine is inactivated and is considered safe for pregnant women. It is reformulated each year to include the anticipated viral strains of the upcoming influenza season.

Pregnant women also should be considered at increased risk from influenza infection in the event of pandemic influenza. Vaccination of pregnant women not only benefits the woman herself but also indirectly confers immunity to her infant, which can last the first 6 months of life when vaccination is not approved for children (*36*). During

a pandemic, an effective vaccine may initially be unavailable or in limited supply. In such a situation, chemoprophylaxis will be an important option for pregnant women. Unfortunately, no information is available regarding the effects on the fetus of neuraminidase inhibitors (oseltamivir and zanamvir), the medications likely to be useful in an H5N1 pandemic (*36*). Thus, weighing the risks associated with infectious exposure in a pregnant woman and risks associated with medication exposure to her unborn child is difficult.

The anthrax attacks of 2001 prompted the first, large-scale recommendations for use of prophylactic medications in response to bioterrorism. The recommended medication for initial antimicrobial drug prophylaxis of asymptomatic exposed adults was ciprofloxacin, with doxycycline and amoxicillin as alternative therapies if the strain was susceptible (*37*). Because of an observed association between fluoroquinolones and joint and cartilage toxicity in juvenile animals (*38*), ciprofloxacin is generally not recommended during pregnancy if efficacious alternatives are available. Although information on the safety of ciprofloxacin in pregnant women was lacking, the available limited information suggested that its use during pregnancy was unlikely to be associated with a high risk for structural birth defects. In addition, maternal exposure to tetracyclines, which include doxycyline, carries the known risks of staining the primary teeth, concern about bone growth and abnormal tooth enamel in the fetus (*39*), and rare instances of hepatic necrosis in pregnant women. Although penicillins are considered safe during pregnancy, the fact that *Bacillus anthracis* strains may have penicillinase activity led to the recommendation that amoxicillin be used for prophylaxis only if the specific strain was shown to be penicillin sensitive. On the basis of these considerations, CDC recommended that ciprofloxacin be the antimicrobial drug of choice for initial prophylactic therapy of asymptomatic pregnant women exposed to *B. anthracis* during the 2001 anthrax attacks (*40*). The American College of Obstetricians and Gynecologists Committee on Obstetric Practice endorsed these recommendations and emphasized that prophylaxis be limited to women exposed to a confirmed environmental contamination or a high-risk source, as determined by local public health officials (*41*).

In 2003, the United States embarked on an effort to vaccinate public health and medical bioterrorism response teams against smallpox. In the absence of circulating smallpox virus, vaccination in pregnant women or women who desire to become pregnant within 28 days of the vaccination is contraindicated because of the risk for fetal vaccinia (*30*). However, after an intentional attack, pregnancy should not be considered an absolute contraindication to vaccination (*30*). In the event of exposure or high risk for exposure to smallpox, pregnant women are advised to receive the vaccine because the risk for death and serious illness from smallpox (particularly during pregnancy) outweighs the risk for fetal vaccinia.

Despite the recommendations that pregnant women avoid vaccination, several pregnant women were inadvertently vaccinated during the smallpox vaccination campaign and were encouraged to enroll in the National Smallpox Vaccine in Pregnancy Registry (*42*). Preliminary results from the registry suggest that the rates of pregnancy loss, preterm birth, and birth defects among infants born to vaccinated women did not increase, but evaluation is ongoing. Pregnancy registries such as this and the Department of Defense Birth and Infant Health Registry (*43*) should be considered whenever emergency response activities invoke the use of medications or vaccines with unknown effects on pregnant women and fetuses.

These examples demonstrate some of the challenges faced by pregnant women and their healthcare providers when considering prophylaxis and treatment in response to emerging infections or bioterrorism attacks. In most instances, information on the effects of the medication or vaccine on the fetus is limited. Decisions regarding appropriate prophylaxis and treatment of pregnant women must take into account the risks associated with specific medications or vaccines versus the risk for illness and death from a possible infectious exposure.

Conclusions

Developing recommendations for prophylaxis and treatment of pregnant women infected with emerging and bioterrorism pathogens can be especially difficult. Data on the effects of some emergency response countermeasure treatments on pregnant women and fetuses are limited. Emergency response planners should include recommendations for pregnant women in pre-event response plans, rather than creating them during an emergency. Clinicians should become familiar with pregnancy-related recommendations for prophylaxis and treatment of persons with emerging and bioterrorism pathogens so that they are prepared to discuss risks and benefits of recommended treatments with their pregnant patients. In an emergency response setting, pregnant women should be encouraged to consider their own health and safety and the effect of potential ill health on their pregnancy, should be offered prenatal evaluation for fetal abnormalities if desired, and should be encouraged to enroll in pregnancy registries when applicable. Long-term goals should include evaluation of the effects of emergency response treatments for the pregnant woman and fetus, and research and development of safer and effective medications when warranted.

Dr Cono is a pediatrician and a medical epidemiologist at the Centers for Disease Control and Prevention. Her research interests include emerging infectious diseases, vaccine science, and terrorism preparedness.

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Emerging Infections and Pregnancy

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A key component of the response to emerging infections is consideration of special populations, including pregnant women. Successful pregnancy depends on adaptation of the woman's immune system to tolerate a genetically foreign fetus. Although the immune system changes are not well understood, a shift from cell-mediated immunity toward humoral immunity is believed to occur. These immunologic changes may alter susceptibility to and severity of infectious diseases in pregnant women. For example, pregnancy may increase susceptibility to toxoplasmosis and listeriosis and may increase severity of illness and increase mortality rates from influenza and varicella. Compared with information about more conventional disease threats, information about emerging infectious diseases is quite limited. Pregnant women's altered response to infectious diseases should be considered when planning a response to emerging infectious disease threats.

As strategies to deal with emerging infectious disease threats are developed, a key component is consideration of special populations, including pregnant women (*1*). Several issues are relevant to infectious disease threats during pregnancy. First, changes in immunity and physiology during pregnancy may make pregnant women more susceptible to or more severely affected by infectious diseases. Second, the effects of infectious diseases on the fetus may be unknown and difficult to predict, and diagnosis of infection in the fetus or infant can be challenging. Third, prophylaxis and treatment appropriate for the general population might not be appropriate for pregnant women. We focus on the first of these considerations: the immunology of pregnancy and the effects of emerging infectious diseases on the pregnant woman.

Although knowledge of the immunology of pregnancy has evolved tremendously over the past decade, many unanswered questions remain, such as how immune function is altered during pregnancy and how this alteration may affect susceptibility to and severity of infectious diseases. Although the effects of some infectious agents during pregnancy are well known, knowledge about many others is limited. A challenge to the study of infectious diseases during pregnancy is the selection of an appropriate control group; many studies have been retrospective and without control groups. Compared with knowledge about more conventional infectious disease threats, knowledge about novel and emerging infectious diseases during pregnancy is even more limited. Such lack of knowledge causes concern, given that an altered response to infectious diseases during pregnancy may require altered responses to emerging infectious disease threats. We describe the immunologic changes that may affect the course of infectious diseases in pregnant women, briefly summarize what is known about infectious diseases during pregnancy, and then focus on the particular challenges of dealing with emerging infectious diseases in pregnant women.

Immunology of Pregnancy

One of the most intriguing puzzles in modern immunology involves the "paradox of pregnancy," in which immunologic tolerance to paternally derived fetal antigens is achieved despite an apparently adequate maternal defense against infection. With 50% of its genetic material derived from its father, the fetus's susceptibility to rejection by the maternal immune system is similar to the susceptibility of a transplanted organ. Evidence indicates that the maternal immune system may tolerate fetal antigens by suppressing cell-mediated immunity while retaining normal humoral immunity. These changes are known to occur locally at the maternal-fetal interface but may also affect systemic immune responses to infection. Although pregnant women are not immunosuppressed in the classic sense, immunologic changes of pregnancy may induce a state of increased susceptibility to certain intracellular pathogens, including viruses, intracellular bacteria, and parasites.

Maternal-Fetal Interface

The fetal allograft is exposed to the maternal immune system at the placenta and fetal membranes (the amnion

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and chorion), collectively described as the maternal-fetal interface. On the fetal side of the interface, the placenta and membranes enclose the fetus and are derived entirely from fetal tissue. Forming a specialized epithelial surface within the placenta, fetal syncytiotrophoblast cells directly contact maternal blood for nutrient exchange. On the maternal side of the interface, the uterine tissue in contact with the placenta and fetal membranes, the decidua, is rich in specialized maternal immune cells including lymphocytes and macrophages (*2*). Despite the prolonged direct exposure of decidual leukocytes and maternal blood to fetal antigens, the immune system does not recognize the fetus as foreign. Several mechanisms underlie this maternal tolerance of fetal tissues.

Humoral Immunity

Also known as antibody-mediated immunity, humoral immunity results from recognition of pathogens by specific antibodies. Most effective against extracellular pathogens, humoral immunity is essential for fighting many bacterial infections. The bacteria become coated in antibodies, which then mediate uptake of the pathogens by phagocytic cells, including neutrophils and macrophages. Presentation of the bacterial antigens on the surface of the macrophage then stimulates B lymphocytes specific to the pathogen, and the B cells produce more antibodies to control the infection. This humoral immune response is augmented by T-helper type II (Th2) lymphocytes, which provide costimulation and induce replication of the B cells. The Th2 response during pregnancy results in vigorous antibody-mediated immunity to pathogens (*2*).

Cell-Mediated Immunity

Essential for controlling intracellular pathogens, cell-mediated immunity involves lymphocyte recognition of cell-associated foreign antigens, followed by destruction of the infected host cells. In contrast to humoral immunity, this arm of the immune response is stimulated by T-helper type I (Th1) lymphocytes and the cytokines they release. The most important effectors of the cell-mediated immune response, cytotoxic T lymphocytes, are the main immune cells that recognize foreign antigens on the surface of infected "self" cells. Cells infected with viruses or other intracellular pathogens are cytotoxic T lymphocytes' most common targets. The cell-mediated immune response is critical for controlling such pathogens because their intracellular location shelters them from antibody binding.

T-Helper Cells and the Th1-Th2 Shift

Emphasis on cell-mediated immunity versus humoral immunity changes according to the type of T-helper lymphocytes responding to an infectious threat. Multiple factors, including the cytokine environment and costimulatory molecules present during activation of the T-helper cell, determine the development of either Th1- or Th2-helper phenotype. One hypothesis is that, in addition to hormonal factors that affect the Th1-Th2 balance, macrophages present at the maternal-fetal interface release predominantly Th2-stimulating cytokines and contribute to the overall dominance of humoral immunity during pregnancy (*3*). In addition to stimulating B lymphocytes, Th2 cells suppress the cytotoxic T lymphocyte response, decreasing the robustness of cell-mediated immunity. In the uterine decidua, the Th2 cytokine environment favors activation of B lymphocytes, resulting in stimulation of antibody secretion and suppression of cell-mediated immunity (*3*). This phenomenon is often referred to as the Th1-Th2 shift of pregnancy and is thought to contribute to maternal tolerance of the fetus by suppressing the antifetal cell-mediated immune response.

Systemic Immune Changes

An evolving model of pregnancy-associated immune changes suggests that the hormonal environment of pregnancy contributes to local suppression of cell-mediated immunity at the maternal-fetal interface while mediating a systemic change toward Th2 dominance. That the local Th1-Th2 shift may also influence the systemic maternal immune response during pregnancy is evidenced in pregnant patients with autoimmune disorders. Women with rheumatoid arthritis, a predominantly cell-mediated autoimmune disorder, tend to experience remissions during pregnancy (*4*). Similarly, patients with multiple sclerosis have fewer exacerbations while pregnant but worsening symptoms during the postpartum period (*5*). Systemic lupus erythematosis, however, a predominantly antibody-mediated autoimmune disorder, often worsens during pregnancy, perhaps due to increased immunoglobulin synthesis and decreased clearance of immune complexes resulting from robust Th2 activity (*3*,*6*). These well-studied changes in severity of autoimmune disorders during pregnancy illustrate systemic immune alterations that occur in conjunction with the Th1-Th2 shift. Systemic suppression of cell-mediated immunity may contribute to increased susceptibility to some intracellular pathogens—including viruses, bacteria, and parasites—during pregnancy.

Pregnancy and Conventional Infectious Disease Threats

Pregnant women may be more susceptible and more severely affected by several infectious diseases such as malaria and measles. Pregnant women in malaria-endemic regions are at risk of becoming infected with *Plasmodium falciparum*, 1 of 4 parasites that cause malaria in humans (*7*). The increased incidence and severity of malaria may

occur especially in primiparous women. Although parasite density is highest in nonimmune women during their first pregnancy, even a previously immune woman can become more susceptible to malaria infection during pregnancy (*7*). In a 14-year follow-up study of women of reproductive age (15–45 years) in 1 area of the Gambia, McGregor and Smith found a higher prevalence of parasitemia among pregnant women than among nonpregnant women (*8*). Prevalence of infection and parasite density are highest during the first half of pregnancy and decline gradually during the second half (*7*).

Evidence also indicates that measles (rubeola) is more common and severe in pregnant women. Accounts of measles outbreaks before an effective vaccine was available indicate that pregnant women may be more severely affected. For example, the investigation of an outbreak of measles in Greenland in 1951 showed that mortality rates were higher among pregnant women than nonpregnant women. Pregnant women were also more likely to experience heart failure (*9*). A relatively recent outbreak of >1,700 confirmed cases of measles in Houston during 1988–1989 also resulted in a high rate of serious complications among infected pregnant women, which suggests that the outbreak disproportionately affected pregnant women (*10*).

Increased Disease Susceptibility

Pregnancy may be a risk factor for acquiring certain infectious diseases, such as toxoplasmosis, Hansen disease, and listeriosis. *Toxoplasma gondii* is a parasite that infects humans primarily through ingestion of infected raw or undercooked meat and, less frequently, by exposure to infected cat feces. This intracellular pathogen can be transmitted transplacentally to the fetus. A cross-sectional study of 2,242 women in Brazil showed that previous pregnancy was a risk factor for serologic evidence of prior infection with toxoplasmosis (*11*). In a follow-up prospective cohort study, the same investigators found that pregnant women who were seronegative for *Toxoplasma* were more than twice as likely as nonpregnant women to seroconvert; acute infection developed in 8.6% of pregnant women (*12*). These findings are consistent with animal data showing that pregnant mice have lower resistance to *Toxoplasma* than nonpregnant control mice (*13*).

Pregnant women may be more likely to show clinical signs of Hansen disease, or leprosy. The causative agent, *Mycobacterium leprae*, can multiply and cause symptomatic disease, particularly in hosts with decreased immunity. The decreased cell-mediated immunity associated with pregnancy may predispose pregnant women to this disease (*14*). A recent report describes a cohort of 40 patients with Hansen disease in Texas, 3 of whom were pregnant (*14*). In addition to evidence supporting the theory that pregnant women are more susceptible to Hansen disease, evidence exists that pregnant women may be more likely to experience relapse of disease. Among 25 women in an Ethiopian cohort who had been treated and had therapy discontinued when considered cured, almost half (n = 12) experienced a relapse of disease when they became pregnant (*15*).

Listeria monocytogenes, a foodborne pathogen, is responsible for ≈2,500 cases of serious illness in the United States each year. *Listeria* infections are more common during pregnancy; one quarter to one third of all cases of listeriosis occur in pregnant women (*16*,*17*). In 2000, an outbreak of listeriosis among Hispanic persons in North Carolina was reported as a result of ingestion of contaminated homemade Mexican-style cheese; 11 of the 13 cases were in pregnant women (*18*).

Increased Disease Severity

For pregnant women, certain infectious diseases, such as influenza and varicella, may have a more severe clinical course, increased complication rate, and higher case-fatality rate. For example, influenza infections cause more severe illness and higher mortality rates for pregnant women. During the 1918–19 influenza pandemic, the mortality rate was 27% for pregnant women, higher in the last trimester, and it increased to 50% if pneumonia developed (*19*). Freeman and Barno reported that during the 1957–1958 pandemic, 50% of the deaths from influenza among reproductive-aged women in Minnesota occurred in pregnant women and that influenza was the leading cause of maternal death in Minnesota (*20*). Increased incidence and severity of illness has also been observed during interpandemic periods. In a review of the Tennessee Medicaid program from 1974 through 1993, pregnant women in their third trimester were 3–4 times as likely as postpartum women to be hospitalized for an acute cardiopulmonary condition during influenza season (*21*). In addition to immunologic changes, other physiologic changes in pregnancy such as increased heart rate, stroke volume, and oxygen consumption, and decreased lung capacity may contribute to this increased risk for illness during pregnancy. Due to the high risk for influenza-related complications, women who will be pregnant during the influenza season should be vaccinated (*22*).

Clinical evidence indicates that primary varicella infections during pregnancy tend to be more severe and that varicella pneumonia seems to be more common among pregnant women than among nonpregnant women. For example, in a case-series of 43 pregnant women reported by Paryani and Arvin, pneumonia developed in ≈10%; 2 of these women required ventilatory support and 1 died (*23*). By comparison, the rate of pneumonia as a complication of varicella infection among the general population is

0.3%–1.8% (*24*). Similarly, pregnant women with varicella pneumonia are more likely to die than nonpregnant women with varicella pneumonia. Haake reviewed 34 published cases of untreated varicella pneumonia in pregnant women and found that 12 (35%) died. By contrast, the mortality rate for nonpregnant women with varicella pneumonia is ≈11% (*24*).

Challenges

Emerging infectious diseases, defined as infectious diseases whose incidence in humans has increased during the past 2 decades or threatens to increase in the near future, are increasingly recognized by physicians as an important threat to pregnant women. Emerging infectious diseases include novel pathogens that have newly emerged, such as severe acute respiratory syndrome (SARS), as well as pathogens that could potentially be used as biologic weapons. Unfortunately, information about how pregnant women are affected by many of these novel and emerging infections is limited.

Novel Pathogens

During the worldwide outbreak of SARS in 2003, several countries reported cases in pregnant women. Although these numbers were too small to enable definitive conclusions as to whether SARS was more severe among pregnant than nonpregnant women, some evidence indicates that it may be. The largest case series of pregnant women with SARS was from Hong Kong Special Administrative Region, People's Republic of China, where 12 pregnant women with SARS were admitted to 5 public hospitals; 3 of them died, giving a case-fatality rate of 25% (*25*). In a case-control study conducted in the same region, pregnant women with SARS had more severe disease than nonpregnant women and an increased risk for admission to the intensive care unit, development of renal failure, development of disseminated intravascular coagulopathy, and death (*26*). Of 8 cases of laboratory-confirmed SARS reported in the United States, 2 were in pregnant women; the small number of cases precludes definitive conclusions about the severity of disease (*27*).

Potential Effects of Bioterrorism

The Working Group on Civilian Biodefense has identified a limited number of biologic agents that are of particular concern (*28*). Evidence exists that infection with some of these pathogens, including smallpox virus and some of the hemorrhagic fever viruses, may be more severe during pregnancy.

Clinical experience with smallpox (variola virus) before vaccination and disease eradication indicates that pregnant women are more susceptible to variola infection and have more severe disease (*29*,*30*). Pregnancy is associated with an increased smallpox case-fatality rate; in the large case-series study in India reported by Rao et al., unvaccinated pregnant women were 3 times more likely to die than were nonpregnant women and men admitted to the hospital during the same time period (*29*). Pregnant women are more likely than nonpregnant women to have hemorrhagic smallpox (purpura variolosa), a severe variety of the disease (*30*).

The viral hemorrhagic fevers, including Lassa fever and Ebola, may be more severe during pregnancy. The first reported case of Lassa fever, caused by infection with an arenavirus, was described in a pregnant patient. In this initial outbreak, 11 patients and staff members who were exposed to the index patient died (*31*). The case-fatality rate is higher for pregnant women, particularly in the third trimester, than for nonpregnant women (*31*,*32*). Women who have Lassa fever late in pregnancy have the highest circulating levels of viremia and therefore tend to be the sickest. Evidence indicating that the placenta may be a preferred site for viral replication may help explain why illness and death increase during the third trimester of pregnancy (*32*). One study found that after pregnancy ended, whether by abortion or normal delivery, women rapidly improved (*32*).

Ebola virus, a member of the Filoviridae group, is transmitted by direct contact with blood, secretions, or contaminated objects and is associated with high case-fatality rates (*28*). Investigations of outbreaks in Africa suggest that Ebola infection may be more severe during pregnancy and that mortality rates are higher. Pregnant women infected with Ebola more often have serious complications, such as hemorrhagic and neurologic sequelae, than do nonpregnant patients (*31*). Unlike risk for death from Lassa fever, which is highest during the third trimester of pregnancy, risk for death from Ebola is similar during all trimesters (*33*).

Other Emerging Infections

Pneumocystis jiroveci (formerly *P. carinii*) has long been identified as a cause of pneumonia in immunocompromised persons. *Pneumocytis* pneumonia was first identified in malnourished children in European orphanages during World War II and was later associated with severe immunosuppression in HIV-infected persons (*34*). However, this agent is increasingly causing infection among immunocompetent persons. A mild or asymptomatic form of *P. jiroveci* infection occurs in immunocompetent hosts, and this infection may be more common in pregnant women than in nonpregnant women. In a small pilot study, nasal swabs from 33 healthy women in their third trimester of pregnancy were compared with those from 28 healthy nonpregnant women. *P. jiroveci* DNA was isolated from 5 of the pregnant women and none of the

nonpregnant women (p = 0.04) (*35*), which indicates that the immune changes associated with pregnancy may favor asymptomatic nasal carriage of this organism. Evidence also indicates that *Pneumocytis* pneumonia may be more severe during pregnancy (*35*) and that *Pneumocystis* may be perinatally transmitted by HIV-infected women to their children (*34*).

Psittacosis is primarily a flulike illness characterized by fever, headache, and atypical pneumonia. *Chlamydophila psittaci* (formerly *Chlamydia psittaci*), the causative agent, is transmitted by inhalation of material from infected birds or by exposure to infected amniotic fluid or placentas of sheep or goats. Although each year, ≈75–100 cases of psittacosis occur in the United States, only 14 cases of psittacosis have been reported in pregnant women, including a recent case in a pregnant Montana sheep rancher. Illness during pregnancy can be quite severe, mimicking HELLP (hemolysis, elevated liver enzyme levels, and low platelet count) syndrome but without hypertension. Most women rapidly recover after pregnancy (*36*).

Conclusions

Changes in immune function during pregnancy alter a pregnant woman's susceptibility to and severity of certain infectious diseases. These alterations are particularly problematic because physicians may hesitate to provide prophylaxis or aggressive treatment to pregnant women because of concerns about effects on the fetus. For example, despite the 1997 recommendation that women who would be in their second or third trimester of pregnancy during influenza season receive the inactivated influenza vaccine, among women 18–44 years of age, reports of having received the influenza vaccination during the past 12 months were fewer for pregnant than for nonpregnant women (*37*).

Compared with what is known about conventional disease threats, knowledge about currently recognized emerging infectious diseases is quite limited. Soon we will likely be faced with novel pathogens about which little or nothing is known. Because the effects of emerging infections in pregnant women might differ from those in the general population, pregnancy must be considered a potential risk factor for disease susceptibility as well as for illness and death. Unfortunately, pregnancy issues are often not well addressed in outbreak investigations, ongoing prospective studies, or emergency preparedness planning. Future scientific inquiry and medical investigations must include pregnancy-related issues as a vital component.

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Health Consequences of Child Marriage in Africa

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Despite international agreements and national laws, marriage of girls <18 years of age is common worldwide and affects millions. Child marriage is a human rights violation that prevents girls from obtaining an education, enjoying optimal health, bonding with others their own age, maturing, and ultimately choosing their own life partners. Child marriage is driven by poverty and has many effects on girls' health: increased risk for sexually transmitted diseases, cervical cancer, malaria, death during childbirth, and obstetric fistulas. Girls' offspring are at increased risk for premature birth and death as neonates, infants, or children. To stop child marriage, policies and programs must educate communities, raise awareness, engage local and religious leaders, involve parents, and empower girls through education and employment.

Awareness of reproductive health issues in developing nations is growing. Critical issues are the high prevalence of HIV/AIDS among young people; childbearing by young girls, which can lead to obstetric fistulas and death of the mother; and child marriage.

Child marriage, defined as marriage of a child <18 years of age, is an ancient, worldwide custom. Other terms applied to child marriage include "early marriage" and "child brides." Early marriage is vague and does not necessarily refer to children. Moreover, what is early for one person may be late for another. Child bride seems to glorify the process, implying a celebration and a bride who is happy to start a loving union with her spouse. But for the most part, girl brides do not know—and may have never met—their groom.

In 2002, ≈52 million girls <18 years of age were married. With ≈25,000 girls <18 years being married each day, an estimated 100 million will be married by 2012 (*1*). Child marriages occur most frequently in South Asia, where 48% of women aged 15–24 have been married before the age of 18; these figures are 42% for Africa and 29% for Latin America and the Caribbean (*2*).

Although the definition of child marriage includes boys, most children married at <18 years of age are girls. For example, in Mali the girl:boy ratio of marriage before age 18 is 72:1; in Kenya, 21:1; and even in the United States, 8:1 (*3*–*5*). We therefore focus on the social and health consequences of child marriage for girls. And although we focus on African countries, similar arguments over what drives child marriages, how they affect girls, and how to stop them may be applied to other continents.

United Nations Efforts and National Laws

Since 1948, the United Nations and other international agencies have attempted to stop child marriage. Article 16 of the Universal Declaration of Human Rights states that persons must be at "full age" when married and that marriage should be entered into "freely" and with "full consent." In other words, any country that allows child marriage is committing a violation of human rights (*6*). Articles 1, 2, and 3 of the 1962 Convention of Consent to Marriage, Minimum Age for Marriage, and Registration of Marriages require that countries establish a minimum age for marriage and that all marriages be registered (*7*). Article 16 of the 1979 Convention on the Elimination of All Forms of Discrimination against Women requires minimum ages for marriage to be specified and says that child marriages are illegal (*8*). However, not until 1989, at the Convention on the Rights of the Child, did international law define children as persons <18 years of age (Article 1) (*9*). In 1994, the International Conference on Population and Development stated that the minimum age of marriage should be raised and enforced, all forms of coercion and discrimination should be eliminated, marriage should be entered into with free consent and as equal partners, and the education and employment of girls should be encouraged (Principle 9, Action 4.18, Action 5.5) (*10*).

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In many countries, the legal age for marriage is 18, yet some governments enforce these laws loosely. For example, the percentage of girls married before age 18 in Niger is 77%, in Chad 71%, in Mali 63%, in Cameroon 61%, and in Mozambique 57% (*1*). In parts of Ethiopia, 50% of girls are married before the age of 15, and in Mali, 39%. Some marriages even occur at birth; in such instances, the girl is sent to her husband's home at the age of 7 (*11*).

Incentives for Perpetuating Child Marriages

Poverty plays a central role in perpetuating child marriage. Parents want to ensure their daughters' financial security; however, daughters are considered an economic burden. Feeding, clothing, and educating girls is costly, and girls will eventually leave the household. A family's only way to recover its investment in a daughter may be to have her married in exchange for a dowry. In some countries, the dowry decreases as the girl gets older, which may tempt parents to have their daughters married at younger ages. These are not necessarily heartless parents but, rather, parents who are surviving under heartless conditions. Additionally, child marriages form new alliances between tribes, clans, and villages; reinforce social ties; and stabilize vital social status.

Parents worry about ensuring their daughters' virginity and chastity. Child marriage is also seen as a protective mechanism against premarital sexual activity, unintended pregnancies, and sexually transmitted diseases (STDs). The latter concern is even greater in this era of HIV/AIDS.

Girls who marry young tend to be from poor families and to have low levels of education. If they marry men outside their village, they must move away. Coping with the unfamiliar inside and outside the home creates an intensely lonely and isolated life. As these girls assume their new roles as wives and mothers, they also inherit the primary job of domestic worker. Because the husband has paid a hefty dowry, the girl also has immediate pressure to prove her fertility. Girls often embrace their fate and bear children quickly to secure their identity, status, and respect as an adult. As a result, these young girls have high total fertility rates but have missed the opportunities to be children: to play, develop friendships, bond, become educated, and build social skills.

Characteristics of the men who marry young girls are also fairly homogenous. Because men have to pay large dowries for girls, many must work for years to generate enough income. As a result, they are older when they marry, which means that they have little in common to discuss with their young wives except household responsibilities and child rearing. Men also are expected to have had multiple sex partners and to be sexually experienced. Because men are aware of the HIV/AIDS danger, they seek even younger, virginal brides, who are presumably not infected.

Risk for HIV and Other Sexually Transmitted Diseases

A common belief is that child marriage protects girls from promiscuity and, therefore, disease; the reality is quite different. Married girls are more likely than unmarried girls to become infected with STDs, in particular HIV and human papilloma virus (HPV). In sub-Saharan Africa, girls ages 15–19 years are 2–8 times more likely than boys of the same age to become infected with HIV (*12*). The risk of acquiring HIV from a single act of unprotected vaginal intercourse is 2–3 times greater for women than men (*13*). Globally, the prevalence of HIV infections among women is highest from ages 15 to 24; the risk for men peaks 5–10 years later (*12*).

Marriage by age 20 has become a risk factor for HIV infection for young and adolescent girls (*13*), as has been shown by several studies of African populations (*14–16*). A study in Kenya demonstrated that married girls had a 50% higher likelihood than unmarried girls of becoming infected with HIV. This risk was even higher (59%) in Zambia. In Uganda, the HIV prevalence rate for girls 15–19 years of age was higher for married (89%) than single girls (66%); for those 15–29 years of age, HIV prevalence was 28% for married and 15% for single girls. This study noted that the age difference between the men and their wives was a significant HIV risk factor for the wives (*16*). All of these studies showed that girls were being infected by their husbands. A hypothesis relevant to this finding is that a young girl may be physiologically more prone to HIV infection because her vagina is not yet well lined with protective cells and her cervix may be more easily eroded. Risk for HIV transmission is also heightened because hymenal, vaginal, or cervical lacerations increase the transmission rate, and many of these young girls lose their virginity to HIV-infected husbands. Also, STDs such as herpes simplex virus type 2 infection, gonorrhea, or chlamydia enhance girls' vulnerability to HIV (*17–19*).

Another study explored why married girls in Kenya and Zambia had a higher risk for HIV infection. This study concluded that because married girls are under intense pressure to prove their fertility, they have more unprotected intercourse. The study also found that husbands were substantially older (5–14 years) than their wives and were 30% more likely than boyfriends of single girls to be HIV infected. Because of their age alone, the husbands had already had numerous sex partners. Additionally, in these areas of Africa, polygamy is common (*20*).

One fundamental difficulty with child marriage is that girls are financially dependent on their husbands and therefore lack the power to make demands upon them. They cannot ask their husbands to get an HIV test; they cannot abstain from intercourse or demand condom use (*20*); they cannot insist that their husbands be monogamous; and

ultimately, they cannot leave because they cannot repay their high dowry (*21*). In addition, returning to their parents' home may not be an option because divorce is considered unacceptable and leaving their husbands may have serious implications on the social or tribal ties that were developed during the marriage.

Cervical Cancer

Child marriage and polygamy play an important role in another deadly disease, cervical cancer. HPV infection has become endemic to sub-Saharan Africa (*22–24*). Although many African nations do not have the capacity to adequately or effectively screen for cervical cancer or HPV, the incidence of cervical cancer in Africa is estimated to be extremely high. Common risks for cervical cancer are child marriage, low socioeconomic status, poor access to health care, and husbands who had multiple sex partners. For example, in Mali, cervical cancer is the most common cancer in women, has an age-standardized incidence rate of 24.4 per 100,000, and is the second most common cause of death from cancer (*25*). In a case-control study of 200 participants with and without cervical cancer, among whom the mean age at marriage was 15 years, HPV was detected in 97% of the cases and 40% of the controls. The risk factors identified were child marriage, high parity (>10 children), polygamous husbands (>2 wives), and poor genital hygiene (no tap water available and reuse of sanitary napkins). Another study in Morocco had similar findings (*26*), with cervical cancer risk factors identified as child marriage, high parity, long-term use of oral contraceptives, and poor genital hygiene (control participants bathed more frequently, and case-participants used homemade sanitary napkins more frequently). Other studies have also implicated hygiene as a possible factor (*22*,*27*).

Children Bearing Children

Pregnancy poses many challenges for young girls. Because pregnancy suppresses the immune system (*28*), pregnant girls are at increased risk of acquiring diseases like malaria. Malaria kills >1 million people each year, 90% of them in Africa. Approximately 25 million pregnant women are exposed to malaria per year, and pregnant women are among the most severely affected by malaria. About 10.5 million become infected during their second or third trimester (*29*), and among these, the mortality rate is ≈50% (*30*). Not only are pregnant women most susceptible to malaria during their first pregnancy (*31*), but they also have higher rates of malaria-related complications (predominantly pulmonary edema and hypoglycemia) and death than do nonpregnant women. Malaria parasite density is significantly higher in pregnant girls <19 years than in pregnant women >19 years. (*32*) However, a woman who has had malaria during pregnancy is less susceptible to malaria during subsequent pregnancies, unless the woman is also HIV infected (*31*).

The interaction between HIV and malaria in young married girls is devastating. Rates of coinfection are highest in Central African Republic, Malawi, Mozambique, Zambia, and Zimbabwe, where >90% of the population are exposed to malaria and >10% are HIV positive. HIV-infected patients are much more susceptible to infection with *Plasmodium falciparum*. Pregnant women have high malaria parasitemia in the placenta and more severe clinical disease, which affects not just the first pregnancy but all subsequent pregnancies. HIV-infected patients also do not respond as well to standard antimalaria treatment. Finally, malaria increases HIV viral load and raises the risk for mother-to-child HIV transmission (*29*). The biologic interaction between these diseases not only complicates treatment in an already challenging setting but also presents a serious risk for death to pregnant girls <19 years of age.

Children Delivering Children

Births resulting from child marriages are said to be "too soon, too close, too many, or too late" (*33*). For example, a high percentage of girls in Ethiopia (25%), Uganda (42%), and Mali (45%) have given birth by the age of 18 compared with only 1% in Germany, 2% in France, and 10% in the United States (*1*). The problem with children delivering children is that the young mothers are at a significantly higher risk than older women for debilitating illness and even death. Compared with women >20 years of age, girls 10–14 years of age are 5–7 times more likely to die from childbirth, and girls 15–19 years of age are twice as likely (*34*). For example, in Mali, the maternal mortality rate for girls aged 15–19 is 178 per 100,000 live births and for women aged 20–34, only 32 per 100,000. In Togo, for the same age groups, these rates are 286 and 39, respectively (*1*). Reasons for these high death rates include eclampsia, postpartum hemorrhage, HIV infection, malaria, and obstructed labor. Obstructed labor is the result of a girl's pelvis being too small to deliver a fetus. The fetus's head passes into the vagina, but its shoulders cannot fit through the mother's pelvic bones. Without a cesarean section, the neonate dies, and the mother is fortunate if she survives. If sepsis or hemorrhage does not occur and the girl does survive, the tissue and bones of the neonate will eventually soften and the remains will pass through the vagina.

Many times, obstructed labor leads to fistulas; the pressure of the fetal head on the vaginal wall causes tissue necrosis, and fistulas develop between the vagina and the bladder or rectum after the necrotic tissue sloughs. More than 2 million adolescents are living with fistulas, and fistulas develop in ≈100,000 more each year (*35*). Girls ages 10–15 years are especially vulnerable because their pelvic

bones are not ready for childbearing and delivery. Their risk for fistula is as high as 88% (*36*). Once a fistula is formed, fecal or urinary incontinence and peroneal nerve palsy may result and may lead to humiliation, ostracism, and resultant depression. Unless the fistula is surgically repaired, these girls have limited chances of living a normal life and bearing children.

Effects on Offspring

Child marriage affects more than the young girls; the next generation is also at higher risk for illness and death. Adolescent mothers have a 35%–55% higher risk than older women for delivering infants who are preterm and of low birthweight. Mortality rates are 73% higher for infants born to mothers <20 years of age than for those born to older mothers (*37*). The infant mortality rates in Mali are 181 per 1,000 children born to women <20 years and 111 per 1,000 born to mothers ages 20–29 years; in Tanzania these rates are 164 and 88, respectively (*1*). These deaths may be partly because the young mothers are unhealthy, immature, and lack access to social and reproductive services. Their babies are also at high risk of acquiring HIV at delivery and during breastfeeding. Mothers who have had malaria are at increased risk for premature delivery, anemia, and death. Untreated STDs such as gonorrhea, chlamydia, syphilis, and herpes simplex virus infection can have deleterious effects on neonates, such as premature delivery, congenital neonatal infections, and blindness. Even the mortality rate for children <5 years can be 28% higher for children born to young mothers than for those born to mothers >20 years (*38*).

Discussion

Child marriage has far-reaching health, social, economic, and political implications for the girl and her community. It truncates a girl's childhood, creates grave physical and psychological health risks, and robs her of internationally recognized human rights. Ending child marriage requires the consent of all those involved, including fathers and religious, community, and tribal leaders. To break the cycle of poverty, programs are needed to educate and empower women. In 2000, eight Millennium Development Goals outlined a vision that committed member countries to eradicate extreme poverty and hunger, educate all children through primary school, empower women, reduce childhood death, improve mothers' health, combat HIV/AIDS and malaria, ensure environmental sustainability, and develop a global partnership for development by the year 2015. Most of these goals directly affect child marriage. Data show that improvements are being made and that sub-Saharan Africa has the most obstacles to overcome (*39*).

In some countries, child marriage has been declining. Increasing mean age for marriage often results in part from overall advancement of an economy. In some countries, such as Korea, Taiwan, and Thailand, decreasing poverty effectively decreased child marriage by enabling these countries to improve education, increase employment, and provide better health care for the whole nation. Education is a key factor for delaying first sexual activity, pregnancy, marriage, and childbearing. Programs that specifically focused on the status of girls may have directly or indirectly reduced the number of child marriages. Successful programs have provided economic and educational opportunities to young women and their families by employing girls with the specific goal of delaying marriage (*40*), giving families financial incentives to keep their daughters in school (*1*), or feeding children during school to decrease families' expenses. Keeping girls in school or vocational training not only helps protect them from HIV infection, pregnancy, illness, and death but also enhances their earning potential and socioeconomic status. Educated girls can contribute to the health and welfare of their family and marry men of their own choosing and age.

Lack of enforcement renders laws against child marriage ineffective. Through media campaigns and educational outreach programs, governments need to take responsibility for stopping this practice. Local, regional, and national governments can also implement health outreach programs for girls and boys. Learning about reproductive and sexual health, STD prevention, contraception, AIDS, and how to seek health care helps girls negotiate safer sex. Governments must incorporate preventive and treatment programs for reproductive health issues into their health services. Necessary preventive services include supplying mosquito netting and condoms; educating patients about contraceptive methods; providing diagnostic screening for HIV and HPV; and offering treatment options such as medications, cesarean sections, and postpartum care.

Ending child marriage requires a multifaceted approach focused on the girls, their families, the community, and the government. Culturally appropriate programs that provide families and communities with education and reproductive health services can help stop child marriage, early pregnancies, and illness and death in young mothers and their children.

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Anatidae Migration in the Western Palearctic and Spread of Highly Pathogenic Avian Influenza H5N1 Virus

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During the second half of 2005, highly pathogenic avian influenza (HPAI) H5N1 virus spread rapidly from central Asia to eastern Europe. The relative roles of wild migratory birds and the poultry trade are still unclear, given that little is yet known about the range of virus hosts, precise movements of migratory birds, or routes of illegal poultry trade. We document and discuss the spread of the HPAI H5N1 virus in relation to species-specific flyways of Anatidae species (ducks, geese, and swans) and climate. We conclude that the spread of HPAI H5N1 virus from Russia and Kazakhstan to the Black Sea basin is consistent in space and time with the hypothesis that birds in the Anatidae family have seeded the virus along their autumn migration routes.

The spread of highly pathogenic avian influenza (HPAI) H5N1 virus during 2003–2004 in eastern and southeastern Asia, and, in 2005–2006 westward across Asia into Europe, the Middle East, and Africa is not typical of other HPAI epizootics. Until recent events, HPAI outbreaks or epizootics were assumed to first require transmission of a low pathogenic avian influenza (LPAI) virus from wild birds to domestic poultry (*1*). Preventive measures thus focused on surveillance and control in poultry and on stopping transmission to noninfected premises. Usually this strategy successfully extinguished an outbreak, often within the year (*2*). The spread of the disease back to wild birds from domestic fowl was considered relatively rare. The HPAI H5N1 virus is unusual in that virus infections in wild bird populations may cause a high proportion of deaths (*3*–*5*). A list of species that have been infected with HPAI H5N1 virus can be found in online Appendix Table (available at http://www.cdc.gov/ncidod/eid/vol12no11/06-0223-appT.htm). The virus also is persisting in Asia longer than most previous HPAI epizootics, which suggests a local reservoir (*6*,*7*).

Epidemiologic studies during 2004–05 mainly focused on HPAI H5N1 virus persistence in relation to the agroecology of poultry and duck production systems (*6*), and little attention was paid to the role of wild birds in local viral persistence or long-distance spread during that period. Because of 3 major events during mid- to late 2005, wild birds are now suspected of spreading the HPAI H5N1 virus over long distances through migration (*8*,*9*). First, in May 2005, a major HPAI HN51 virus outbreak was discovered in wild birds in Lake Qinghai (western People's Republic of China), an important breeding place for migratory bird species in eastern Asia. Within a few weeks, several hundred birds, mainly bar-headed geese, had died of the disease (*4*,*10*). Eventually, other bird species also became affected, and several virus introductions may have occurred. Soon after the Lake Qinghai episode, HPAI H5N1 virus was detected in wild birds in Mongolia, to the north of Lake Qinghai along the central migration route, and in an area where domestic poultry were scarce (*11*). Second, in October 2005, HPAI H5N1 virus spread westward across Eurasia; outbreaks were recorded in Turkey, Romania, and Ukraine, usually in or near known wintering sites for migratory birds. This long-distance spread took place through areas with no record of any virus presence. Third, in spring 2006, the virus infected large numbers of mute swans and other wild bird species across western Europe, also in areas where no outbreaks had previously

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been detected in domestic poultry, despite continual and intensive surveillance. These incursions occurred after unusual waterfowl movements that were associated with a spell of cold weather in the Black Sea area where HPAI H5N1 virus is believed to have been endemic since autumn 2005.

Arguments may also be raised against the hypothesis that HPAI H5N1 virus is transmitted by wild birds. Invariably, wild birds found to be infected with the virus were either dead or moribund and may not have been able to spread the virus over long distances. Furthermore, in several cases, no straightforward match was found between the appearance of the virus and the presence of the wild birds suspected of spreading it. For example, HPAI H5N1 virus outbreaks that took place in Russia and Kazakhstan during summer 2005 were distributed along important trade routes that link western People's Republic of China to Russia (*12*), rather than any direct migration pathway.

We document and discuss the possible role of migratory birds in the spread of HPAI H5N1 virus during the second half of 2005, on the basis of information and data concerning the role of waterfowl in the ecology of avian influenza viruses; the pattern of Anatidae bird migrations across the western Palearctic, and contemporary, satellite-derived temperature data.

Role of Migratory Waterfowl in Ecology of Avian Influenza Viruses

Avian influenza viruses (AIVs) have been recorded in most bird families (*3*), but the prevalence and diversity of AIV subtypes is not evenly distributed among them (*13*). AIVs have been isolated in 12 bird orders, but most isolations have been reported in the orders Anseriformes (in particular in the family Anatidae: ducks, swans, geese) and Charadriiformes (shore birds, gulls, terns). Although a wide variety of AIV subtypes have been isolated from Charadriiformes (*13*,*14*), they are believed to belong to a somewhat different genetic pool from those isolated in Anseriformes (*15*).

Species from the Anatidae family, in particular, the Anatinae subfamily (ducks), represent the highest risk for transmission to domestic poultry (*16*,*17*) for the following reasons: 1) Anatids harbor the most diverse and highest prevalence of avian influenza viruses (*13*,*14*); 2) historical outbreaks of HPAI in poultry have been linked mainly to strains circulating in ducks, rather than in members of other species (*18*,*19*); 3) domestic ducks (mallards) can excrete large amounts of HPAI H5N1 virus while remaining relatively healthy and are thus able to move the virus across large distances (*7*); and 4) direct contacts between wild anatids and domestic aquatic poultry are believed to be relatively more common than with other groups of wild birds (*20*).

Most waterfowl migrate seasonally, to exploit temporary feed resources during spring and summer, while escaping harsh winter conditions (*21*). An important evolutionary incentive for these migrations is the prolific spring growth in the Arctic, which provides plants and insects rich in the calcium and protein required for egg production (the female mallard duck produces 8–12 eggs) and juvenile growth (*22*). However, the favorable season for breeding is very short in these higher latitudes, and migratory bird populations soon start migrating southward with their juveniles to escape the frosts that occur from midsummer onward (*23*). This frequently results in premigration concentrations of many species of waterfowl south of the breeding areas, where juvenile maturation and adult molting take place before the main southward migration in the autumn. This seasonal aggregation mixes many species with high densities of immunologically naive juveniles alongside adult birds, which are unable to fly for up to 1 month while they molt; this setting is ideal for AIV transmission and redistribution. Previous work on AIV ecology has shown that premigration concentrations of waterfowl, together with the high recruitment rate of immunologically naive juveniles, induce a seasonally and geographically distinct pattern in AIV prevalence with peaks observed just before autumn migrations in interspecies concentration areas (*13*,*24*). During the subsequent southwest wards migration, AIV prevalence declines as a result of increased flock immunity and progressive dispersal of bird populations (*13*,*24*,*25*). Although AIV is more difficult to detect in waterfowl during winter and spring, several studies reviewed by Stallknecht and Shane (*13*) reported that AIV isolates persist until spring. De Marco et al. (*26*) demonstrated that AIV circulated continually from November to March in wintering areas. The high level of flock immunity and the relatively low level of AIV isolation during winter and spring raise the question of AIV survival during this time in the annual cycle (*13*). The survival of the virus in water and ice (*27*) may play a critical role, in terms of virus persistence and in terms of facilitating fecal-oral AIV transmission (*16*). The possible overwintering of AIV in shallow and cold water (*28*) in the Pan-Arctic region and the concentrations during postsummer-breeding and transmission of AIV between subpopulations and bird species during premigration may help sustain the natural AIV cycle. A redistribution of AIV among birds that use different migration routes may well contribute to the survival of the virus across a wide geographic range.

Anatidae Migration Patterns in the Western Palearctic

Northeastern Russia and Siberia are major breeding areas for many migratory Anatidae species in the Palearctic. Birds arrive during the spring, traveling different routes

from Europe, Asia, and Africa. Of particular importance is the west Siberian lowlands (WSL), which has an area of 2,745,000 km^2 and is by far the largest wetlands in the world (Figure 1). WSL is an important breeding area, along with several other large wetlands located in northwestern Russia and northern Scandinavia (Figure 1). In western Europe, the main wetlands that support wintering waterfowl are found along the coastal areas of Denmark, the Netherlands, United Kingdom, France (the Rhône delta), Spain, and northern Italy. In central Europe and western Asia, major wetland areas are found around the Black Sea in Ukraine, Romania (the Danube delta), and Turkey; around the Caspian Sea in Russia and Iran; and in the southeastern part of Iraq. Three recognized routes, or flyways, connect breeding areas to wintering areas in the western Palearctic (*29*) are shown on the overlay of individual species flyways in Figure 2A. The North Sea flyway joins the wetlands of northwestern Russia to western Europe wintering sites and runs through Scandinavia, the Baltic basin, and the North Sea. The Black Sea and Caspian Sea flyways run from the WSL, leading to Mediterranean Europe and western Asia, respectively. When weighted according to the number of birds that use them (Figure 2B), the North Sea flyway stands out as the most important, followed by the Black Sea; the Caspian Sea flyway is of least consequence.

Migration Patterns and Spread of HPAI H5N1 Virus across the Western Palearctic

During July and August 2005, several HPAI H5N1 virus outbreaks were reported in Russia and Kazakhstan (Figure 1). These occurred in domestic poultry, but the strains were genetically related to the Lake Qinghai strain (*30*). Early in October 2005, HPAI H5N1 virus was first encountered in wild fowl and in poultry in Turkey and Romania and in dead swans in Croatia. Again, the sequenced virus was found to be identical to that from Lake Qinghai. This same virus was detected in Ukraine in December 2005 (*31*).

A mission of the World Organization for Animal Health (OIE) to Kazakhstan established that the first outbreaks were located near important molting sites for migratory waterfowl. This finding is further illustrated in Figure 1, which depicts the WSL breeding area, where the first frost took place as early as July (the pale green pixels). The premigration concentration of wild bird populations for molting takes place just south of the WSL. HPAI H5N1 virus may have been brought into southern Siberia through the poultry trade and related traffic, in particular, through the trans-Siberian commercial route (*12*), and, from there, may have entered the wild bird population. However, the observed patterns of virus circulation are also consistent with the critical steps in AIV transmission and redistribution (*13*), and HPAI H5N1 virus may have already been present in the wild bird population during the 2005 spring season in the WSL or at the molting sites and transmitted from there to domestic poultry at the time of main virus transmission in the premigration concentration areas.

The initial outbreaks of HPAI H5N1 virus in Romania, Turkey, and Ukraine occurred close to wetlands frequented by overwintering migratory waterfowl. These locations were clearly far from any known location where HPAI H5N1 virus had been recorded, while the timing and location match the autumn wildfowl migration ahead of the approaching wave of frost (Figure 1).

Figure 2C illustrates the population-weighted overlay of all Anatidae species flyways that coincide with locations of HPAI H5N1 virus in Russia and Kazakhstan observed during the summer 2005, i.e., the spread to be expected if only the bird species using the Caspian Sea flyway had been responsible for further spread. Figure 2D shows the population-weighted overlay of species flyways in relation

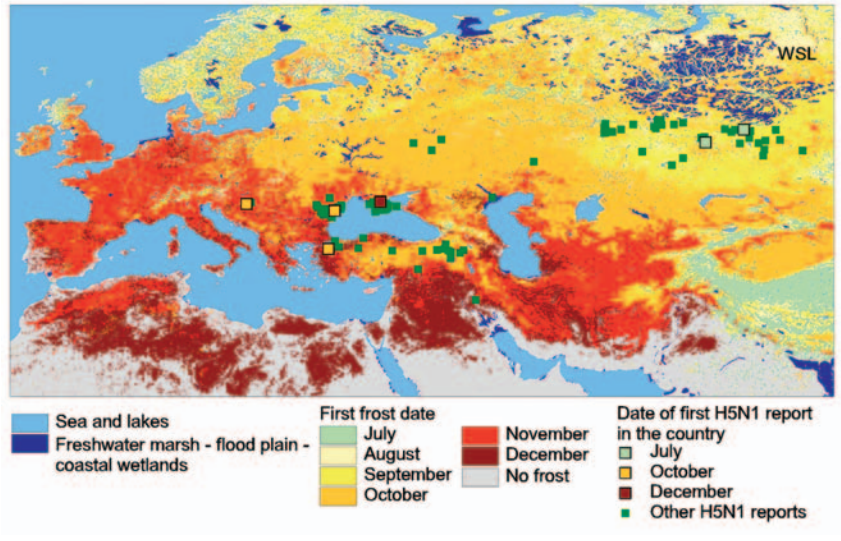

Figure 1. Map showing the spread of highly pathogenic avian influenza (HPAI) H5N1 virus and its environmental context. The background color indicates the month when the first frost was observed, from July through December 2005. The distribution of the main wetlands is indicated (dark blue; west Siberian lowland [WSL]). The reported presence of HPAI H5N1 virus from July 2005 to January 16, 2006, is indicated by squares with color coding for the first report of HPAI H5N1 virus in the country, and by green dots for other records.

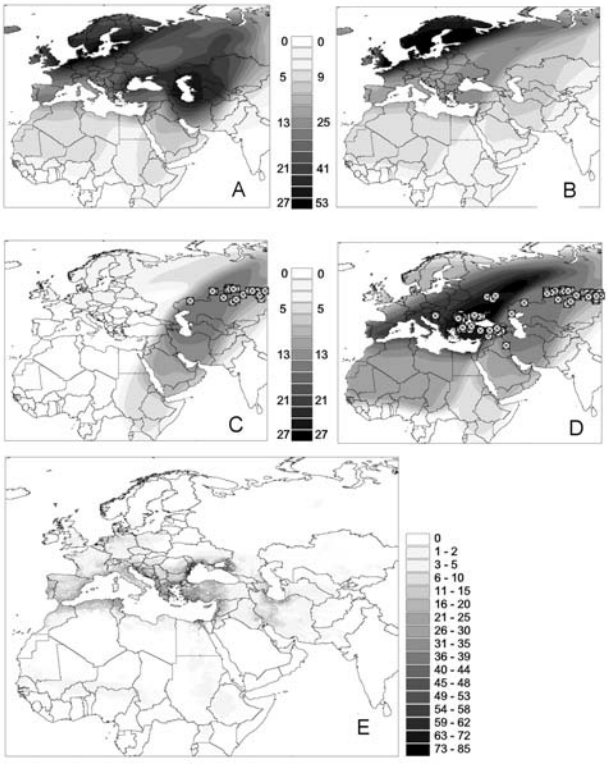

Figure 2. Distribution overlay of migratory flyways of Anatidae bird species in the western Palearctic: each pixel of gray shading indicates the number of species that include the area as part of their flyway. A) All species with an equal weight (indicative of species diversity by pixel). B) Flyways weighted according to their population (indicative of anatid populations). Population-weighted distribution overlay for flyways intersecting highly pathogenic avian influenza (HPAI) H5N1 virus records are shown for C) July through August and D) July through December. E) The maps displayed present the product of map B by the overlay of anatids wintering distribution and by the $\log_{10}$ of poultry density, as an index of the risk for transmission of HPAI H5N1 virus from anatids to domestic poultry in their wintering sites under the affected flyways.

to locations of HPAI H5N1 virus until January 2006. This figure suggests that the Black Sea flyway was also infected if anatids did indeed spread HPAI H5N1 virus through their autumn migration. This apparent discrepancy between HPAI H5N1 virus outbreak locations (Figure 2D) and the pattern of spread that could have been expected from the summer outbreaks locations (Figure 2C) requires further attention. First, the northern limits of the 2 flyways are so close that a figure similar to Figure 2D is obtained just with the summer outbreaks if one simply allows for a 200-km variation in the flyway border or if HPAI H5N1 virus presence is assumed to have occurred 200 km further westward. Second, the boundaries between the flyways are arbitrary; these flyways mainly represent directions taken by subpopulations, representing most diffusive migration paths. A large fraction of wild bird populations distributed across the area with locations of HPAI H5N1 virus reported presence in Russia and Kazakhstan connect to the Black Sea basin (actually, the number of anatids flying from Siberia down to the Black Sea is higher than the number flying to the Caspian Sea basin; Figure 2B). Given the above uncertainties, one may reasonably assume that waterfowl from both flyways may have become infected when they met in premigration concentration areas.

If these flyways are assumed to be used by infected birds, their geographic extent may be used to estimate an index of risk for virus transmission from wildfowl to domestic poultry. The population-weighted overlay of individual waterfowl species' ranges intersecting with HPAI H5N1 virus locations (Figure 2B) can, in turn, be intersected with the wintering areas' boundaries (rescaled from 0 to 1 as an index of wintering site suitability) to delineate the areas where migrating birds are more likely to concentrate. This layer can then be multiplied by overall poultry population density (*32*) to derive a coarse measure of risk for an HPAI H5N1 virus outbreak to occur in domestic poultry (Figure 2E). The resulting display highlights the high-risk areas on the Black Sea coast, in particular, areas adjacent to the wintering areas in Romania, Turkey, and Russia, but also parts of Greece, along the Adriatic coast, the Nile delta, and along the southern edges of the Caspian Sea. A relatively high risk is found across western Europe, which reflects not only the high density of poultry but also the fact that a cluster of 3 westernmost outbreaks in Russia intersect with multiple western European flyways.

Discussion

Our results indicate that the broad-scale pattern of spread of HPAI H5N1 virus from Russia to the Black Sea basin is consistent with the spatial and temporal pattern of Anatidae migration from Siberia. Given that the first recorded signs of HPAI H5N1 virus in Turkey, Romania, and Ukraine took place in the direct vicinity of important waterfowl overwintering sites, Anatidae could have been implicated in the spread of HPAI H5N1 virus to the Black Sea basin. The search for wild bird species carrying HPAI H5N1 virus is in progress and awaits further classification. Several species demonstrably carry the virus without showing clinical signs, as has been recently reported from studies in Russia (*30*) and People's Republic of China (*8*). Most wild birds found dead were geese, swans, and, rarely, wild ducks (when domestic ducks were found infected with, and sometimes dead from HPAI H5N1 virus, this occurred in conjunction with disease outbreaks in terrestrial poultry), which supports the hypothesis that not only mallards but also several other duck species are healthy carriers of HPAI H5N1 virus. The postulate that migratory

anatids can spread the disease over long distances by no means excludes the role of the poultry trade as an important, complementary transmission pathway.

It could be argued that an important contradiction of the hypothesis that wild birds spread HPAI H5N1 virus along their migration paths stems from our "false-positive" predictions (e.g., Figure 2E, Spain, Morocco, Greece). We propose 3 possible explanations for these deviations. First, as well as being along flyways of infected wild bird, establishment of HPAI H5N1 virus in domestic poultry may require additional conditions: 1) an aggregation of waterfowl for a sufficient period (more risk for transmission within wintering areas than at more transient stopover sites), 2) a high proportion of small poultry farms and backyard poultry, and 3) extensive (aquatic) poultry units in contact with waterfowl populations and habitat, i.e., floodplain or other forms of wetland agriculture in close proximity to natural wetlands used as wildfowl wintering sites. Such conditions have been shown to be associated with HPAI H5N1 virus persistence in Southeast Asia (*6*) and were certainly also met in parts of Romania, Turkey, and Ukraine. Second, the overall prevalence of HPAI H5N1 virus found in wild bird populations was very low, usually <1% (*8*). This finding suggests that virus persistence in wild bird populations may be subject to stochastic fluctuation. Also, few infected individual birds are likely to be evenly distributed in the population; i.e., the distribution of infected birds is probably clustered. The scarcity of infected individual birds and their likely clustering produce a pattern in which several regions exposed to equivalent wintering populations may have been exposed to different levels of virus exposure. Finally, HPAI H5N1 virus was found either in dead and apparently healthy ducks, which suggests a dichotomy in wild bird susceptibility. The exact status of species, as sentinels or spreaders, and precise migratory pattern may help explain any inconsistencies that arise from considering all species at equal risk for transmission.

One could also mention here the discrepancies between the geographic spread of HPAI H5N1 virus and overall pattern of wild bird migrations: the virus has never been reported in the Philippines and in several countries farther south such as New Zealand and Australia (although these 2 countries have no migratory anatid populations connecting them to Southeast Asia, they do have many shore bird and wader species in common [*20*]). Conversely, with the possible exception of African countries, HPAI H5N1 virus was established in domestic poultry only in countries connected by flyways with existing infected countries. The introduction in Nigeria is inconclusive. Two species of dabbling ducks, *Anas querquedula* and *A. acuta*, have large wintering concentrations in and near Lake Chad and in the Niger delta, both under the western Siberia/Black Sea flyways, and are presumed to be infected by HPAI H5N1 virus. However, Nigeria imported large numbers of poultry from Turkey and People's Republic of China until a ban was imposed, and illegal trade may well have continued after the ban and brought in infected animals or products (*33*).

The broad approach adopted in this study has clear limitations, given the uncertainties regarding the host range of HPAI H5N1 virus within the Anatidae family, the sizes and distribution of the bird populations, their precise migratory patterns, and the demarcation of the summer and winter habitat. A comprehensive retrospective analysis of HPAI H5N1 virus spread in the western Palearctic would require a better description of the dynamic distribution of wild birds (breeding range, wintering sites, stopover sites, migration pathways) as well as more detailed domestic poultry data (distribution, production structure, species composition, movements through trade) to map the contact points between wild and domestic birds. In addition, local studies could focus on possible introduction points and characterize and detail the specific ecologic conditions in the wild birds–domestic poultry interface that support establishment of the virus, including the local landscape structure (wild bird habitat and farming), climate (e.g., virus survival in the environment), and other agro-ecologic conditions.

Experimental Procedure

Imagery

We used the land surface temperature (LST) data products derived from the Moderate Resolution Imaging Spectroradiometer (MODIS) sensor on board the National Aeronautics and Space Administration's Aqua satellite (*34*). The Aqua satellite acquires daytime images (a local pass time of 1:30 P.M. at the equator) and nighttime images (a local pass time of 1:30 A.M. at the equator). A day/night algorithm was applied to a pair of MODIS daytime and nighttime observations to extract average temperature (when multiple observations are available), and the method yields an accuracy of 1°K with known emissivities (*34*).

Daily LST products were aggregated (averaged) to generate 8-day composite LST product (MYD11A2), and 46 of these 8-day composite LST products are generated per year. The LST product has a spatial resolution of 1 km. We downloaded the 8-day composite LST data (MYD11A2) in 2005 from the US Geological Survey Earth Resources Observation and Science (EROS) Data Center. For each individual 1-km pixel, we analyzed time series data of nighttime LST in 2005 and identified the first 8-day period that experienced frost (LST <0°C) in the fall/winter seasons. We assume that the date of early frost events in

fall/winter seasons is one of many factors that affect the starting date of wild bird migration from north to south.

Distribution Overlays

Distribution data regarding the winter feeding areas and summer breeding areas were extracted from the Global Registry of Migratory Species CD-ROM (*35*). The data on migration flyways were digitized directly from Scott and Rose (*36*). All boundaries were smoothed by a 2.5-decimal degrees filter to avoid sharp edges in distribution boundaries. The population estimates from Delany and Scott (*37*) were assigned to each flyway. In the population-weighted, each flyway contribution was estimated as its relative contribution to the total population of Anatidae along all flyways multiplied by 100 (100× Popflyway/PopAnatidae). The species included in the analysis are the following: *Anas acuta, Anser albifrons, Anser anser, Anser brachyrhynchus, Anas clypeata, Anas crecca, Anser erythropus, Anser fabalis, Aythya ferina, Aythya fuligula, Aythya marila, Aythya nyroca, Anas penelope, Anas platyrhynchos, Anas querquedula, Anas strepera, Branta bernicla, Bucephala clangula, Branta leucopsis, Branta ruficollis, Cygnus columbianus, Cygnus cygnus, Clangula hyemalis, Cygnus olor, Mergellus albellus, Marmaronetta angustirostris, Melanitta fusca, Mergus merganser, Melanitta nigra, Mergus serrator, Netta rufina, Oxyura leucocephala, Polysticta stelleri, Somateria mollissima, Tadorna ferruginea, Tadorna tadorna*. The data on HPAI locations were extracted from HPAI H5N1 virus reported presence, as recorded in the FAO Empres-I database (*31*) between July 1, 2005, and January 16, 2006.

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Dr Gilbert is a postdoctoral fellow at the laboratory of Biological Control and Spatial Ecology in Brussels. He focuses his research on patterns and processes affecting the spatial dynamics of invasive organisms, in particular, the conditions of persistence and spread of highly pathogenic avian influenza in Southeast Asia.

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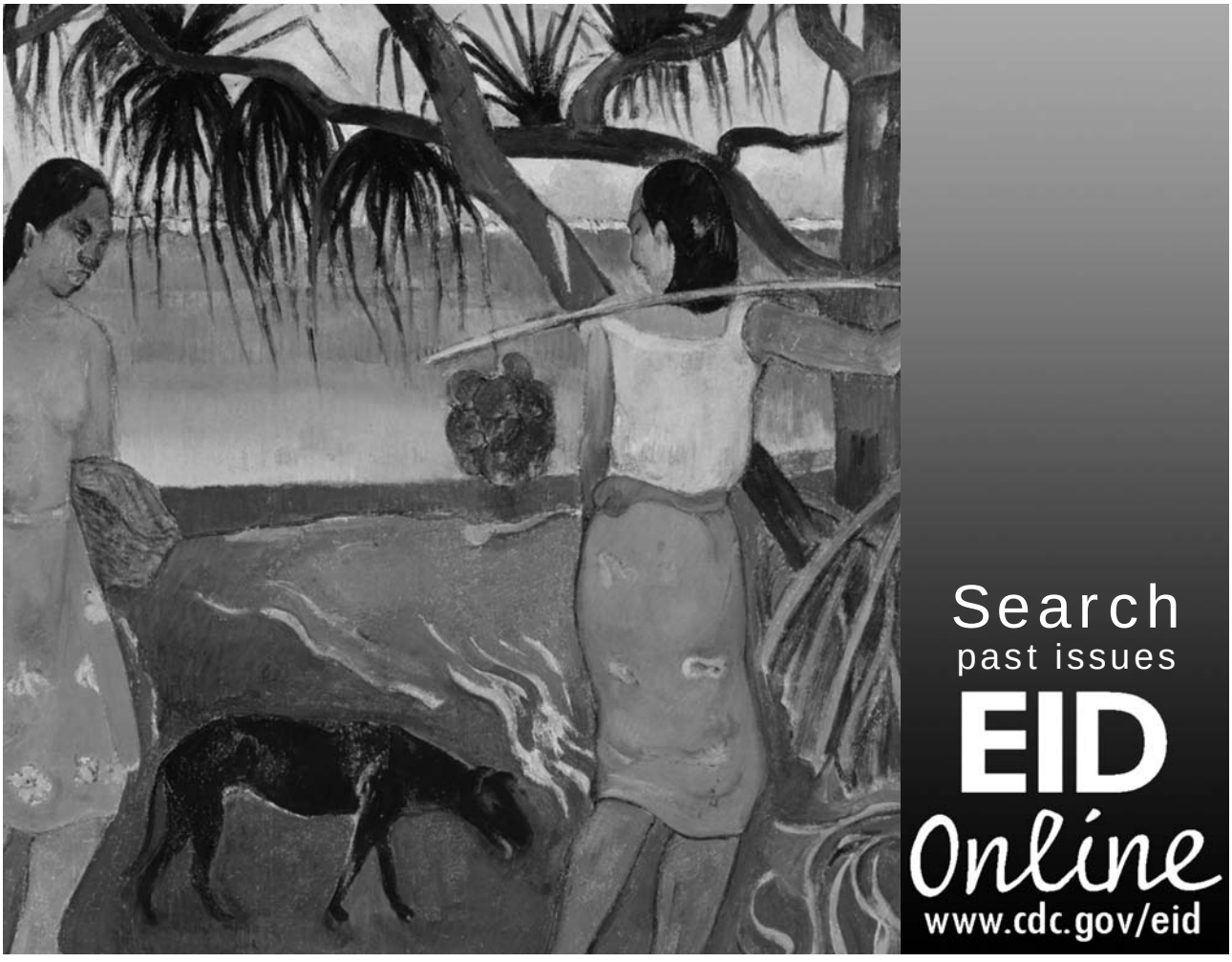

Review of Aerosol Transmission of Influenza A Virus

Raymond Tellier*†

In theory, influenza viruses can be transmitted through aerosols, large droplets, or direct contact with secretions (or fomites). These 3 modes are not mutually exclusive. Published findings that support the occurrence of aerosol transmission were reviewed to assess the importance of this mode of transmission. Published evidence indicates that aerosol transmission of influenza can be an important mode of transmission, which has obvious implications for pandemic influenza planning and in particular for recommendations about the use of N95 respirators as part of personal protective equipment.

Concerns about the likely occurrence of an influenza pandemic in the near future are increasing. The highly pathogenic strains of influenza A (H5N1) virus circulating in Asia, Europe, and Africa have become the most feared candidates for giving rise to a pandemic strain.

Several authors have stated that large-droplet transmission is the predominant mode by which influenza virus infection is acquired (*1*–*3*). As a consequence of this opinion, protection against infectious aerosols is often ignored for influenza, including in the context of influenza pandemic preparedness. For example, the Canadian Pandemic Influenza Plan and the US Department of Health and Human Services Pandemic Influenza Plan (*4*,*5*) recommend surgical masks, not N95 respirators, as part of personal protective equipment (PPE) for routine patient care. This position contradicts the knowledge on influenza virus transmission accumulated in the past several decades. Indeed, the relevant chapters of many reference books, written by recognized authorities, refer to aerosols as an important mode of transmission for influenza (*6*–*9*).

In preparation for a possible pandemic caused by a highly lethal virus such as influenza A (H5N1), making the assumption that the role of aerosols in transmission of this virus will be similar to their role in the transmission of known human influenza viruses would seem rational. Because infection with influenza A (H5N1) virus is associated with high death rates and because healthcare workers cannot as yet be protected by vaccination, recommending an enhanced level of protection, including the use of N95 respirators as part of PPE, is important. Following are a brief review of the relevant published findings that support the importance of aerosol transmission of influenza and a brief discussion on the implications of these findings on pandemic preparedness.

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Influenza Virus Aerosols

By definition, aerosols are suspensions in air (or in a gas) of solid or liquid particles, small enough that they remain airborne for prolonged periods because of their low settling velocity. For spherical particles of unit density, settling times (for a 3-m fall) for specific diameters are 10 s for 100 μm, 4 min for 20 μm, 17 min for 10 μm, and 62 min for 5 μm; particles with a diameter <3 μm essentially do not settle. Settling times can be further affected by air turbulence (*10*,*11*).

The median diameters at which particles exhibit aerosol behavior also correspond to the sizes at which they are efficiently deposited in the lower respiratory tract when inhaled. Particles of ≥6-μm diameter are trapped increasingly in the upper respiratory tract (*12*); no substantial deposition in the lower respiratory tract occurs at ≥20 μm (*11*,*12*). Many authors adopt a size cutoff of ≤5 μm for aerosols. This convenient convention is, however, somewhat arbitrary, because the long settling time and the efficient deposition in the lower respiratory tract are properties that do not appear abruptly at a specific diameter value. Certainly, particles in the micron or submicron range will behave as aerosols, and particles >10–20 μm will settle rapidly, will not be deposited in the lower respiratory tract, and are referred to as large droplets (*10*–*12*).

Coughing or sneezing generates a substantial quantity of particles, a large number of which are <5–10 μm in

diameter [reviewed in (*10*)]. In addition, particles expelled by coughing or sneezing rapidly shrink in size by evaporation, thereby increasing the number of particles that behave as aerosols. Particles shrunken by evaporation are referred to as droplet nuclei (*10–12*). This phenomenon affects particles with a diameter at emission of ≤20 μm, and complete desiccation would decrease the diameter to a little less than half the initial diameter (*10*). Droplet nuclei are hygroscopic. When exposed to humid air (as in the lungs), they will swell back. One would expect that inhaled hygroscopic particles would be retained in the lower respiratory tract with greater efficiency, and this hypothesis has been confirmed experimentally (*11*,*12*). Because aerosols remain airborne, they can be carried over large distances, which may create a potential for long-range infections. The occurrence of long-range infections is affected by several other factors. These include dilution, the infectious dose, the amount of infectious particles produced, the duration of shedding of the infectious agent, and the persistence of the agent in the environment (*11*). Inferring an absence of aerosols because long-range infections are not frequently observed is incorrect.

Humans acutely infected with influenza A virus have a high virus titer in their respiratory secretions, which will be aerosolized when the patient sneezes or coughs. The viral titer measured in nasopharyngeal washes culminates on approximately day 2 or 3 after infection and can reach up to 10^7 50% tissue culture infective dose ($TCID_{50}$)/mL (*13*,*14*). The persistence of the infectivity of influenza virus in aerosols has been studied in the laboratory. In experiments that used homogeneous aerosolized influenza virus suspensions (mean diameter 6 μm), virus infectivity (assessed by in vitro culture) at a fixed relative humidity undergoes an exponential decay; this decay is characterized by very low death rate constants, provided that the relative humidity was in the low range of 15%–40% (*15*,*16*). These results are consistent with those of an older study (admittedly performed in a more rudimentary manner) in which infectious influenza viruses in an aerosol could be demonstrated for up to 24 h by using infection in mice as a detection method, provided that the relative humidity was 17%–24% (*17*). In all these studies, the decay of virus infectivity increased rapidly at relative humidity >40%. The increased survival of influenza virus in aerosols at low relative humidity has been suggested as a factor that accounts for the seasonality of influenza (*15*,*16*). The sharply increased decay of infectivity at high humidity has also been observed for other enveloped viruses (e.g., measles virus); in contrast, exactly the opposite relationship has been shown for some nonenveloped viruses (e.g., poliovirus) (*11*,*15*,*16*).

Experimental Influenza Infection

Experimental infection studies permit the clear separation of the aerosol route of transmission from transmission by large droplets. Laboratory preparation of homogeneous small particle aerosols free of large droplets is readily achieved (*13*,*18*). Conversely, transmission by large droplets without accompanying aerosols can be achieved by intranasal drop inoculation (*13*).

Influenza infection has been documented by aerosol exposure in the mouse model, the squirrel monkey model, and human volunteers (*12*,*13*,*17–19*). Observations made during experimental infections with human volunteers are particularly interesting and relevant. In studies conducted by Alford and colleagues (*18*), volunteers were exposed to carefully titrated aerosolized influenza virus suspensions by inhaling 10 L of aerosol through a face mask. The diameter of the aerosol particles was 1 μm–3 μm. Demonstration of infection in participants in the study was achieved by recovery of infectious viruses from throat swabs, taken daily, or by seroconversion, i.e., development of neutralizing antibodies. The use of carefully titrated viral stocks enabled the determination of the minimal infectious dose by aerosol inoculation. For volunteers who lacked detectable neutralizing antibodies at the onset, the 50% human infectious dose (HID_{50}) was 0.6–3.0 $TCID_{50}$, if one assumes a retention of 60% of the inhaled particles (*18*). In contrast, the HID_{50} measured when inoculation was performed by intranasal drops was 127–320 $TCID_{50}$ (*13*). Additional data from experiments conducted with aerosolized influenza virus (average diameter 1.5 μm) showed that when a dose of 3 $TCID_{50}$ was inhaled, ≈1 $TCID_{50}$ only was deposited in the nose (*12*). Since the dose deposited in the nose is largely below the minimal dose required by intranasal inoculation, this would indicate that the preferred site of infection initiation during aerosol inoculation is the lower respiratory tract. Another relevant observation is that whereas the clinical symptoms initiated by aerosol inoculation covered the spectrum of symptoms seen in natural infections, the disease observed in study participants infected experimentally by intranasal drops was milder, with a longer incubation time and usually no involvement of the lower respiratory tract (*13*,*20*). For safety reasons, this finding led to the adoption of intranasal drop inoculation as the standard procedure in human experimental infections with influenza virus (*13*).

Additional support for the view that the lower respiratory tract (which is most efficiently reached by the aerosol route) is the preferred site of infection is provided by studies on the use of zanamivir for prophylaxis. In experimental settings, intranasal zanamivir was protective against experimental inoculation with influenza virus in intranasal drops (*21*). However, in studies on prophylaxis of natural

infection, intranasally applied zanamivir was not protective (*22*), whereas inhaled zanamivir was protective in one study (*23*) and a protective effect approached statistical significance in another study (*22*). These experiments and observations strongly support the view that many, possibly most, natural influenza infections occur by the aerosol route and that the lower respiratory tract may be the preferred site of initiation of the infection.

Epidemiologic Observations

In natural infections, the postulated modes of transmission have included aerosols, large droplets, and direct contact with secretions or fomites because the virus can remain infectious on nonporous dry surfaces for ≤48 hours (*24*). Because in practice completely ruling out contributions of a given mode of transmission is often difficult, the relative contribution of each mode is usually difficult to establish by epidemiologic studies alone. However, a certain number of observations are consistent with and strongly suggestive of an important role for aerosol transmission in natural infections, for example the "explosive nature and simultaneous onset [of disease] in many persons" (*9*), including in nosocomial outbreaks (*25*). The often-cited outbreak described by Moser et al. on an airplane with a defective ventilation system is best accounted for by aerosol transmission (*26*). Even more compelling were the observations made at the Livermore Veterans Administration Hospital during the 1957–58 pandemic. The study group consisted of 209 tuberculous patients confined during their hospitalization to a building with ceiling-mounted UV lights; 396 tuberculous patients hospitalized in other buildings that lacked these lights constituted the control group. Although the study group participants remained confined to the building, they were attended to by the same personnel as the control group, and there were no restrictions on visits from the community. Thus, it was unavoidable at some point that attending personnel and visitors would introduce influenza virus in both groups. During the second wave of the pandemic, the control group and the personnel sustained a robust outbreak of respiratory illness, shown retrospectively by serology to be due to the pandemic strain influenza A (H2N2), whereas the group in the irradiated building remained symptom free. The seroconversion rate to influenza A (H2N2) was 19% in the control group, 18% in personnel, but only 2% in the study group (*27*,*28*).

Whereas UV irradiation is highly effective in inactivating viruses in small-particle aerosols, it is ineffective for surface decontamination because of poor surface penetrations. It is also ineffective for large droplets because the germicidal activity sharply decreases as the relative humidity increases (*28*). Furthermore, because the installation of UV lights was set up in such a way as to decontaminate the upper air of rooms only, large droplets would not have been exposed to UV, whereas aerosols, carried by thermal air mixing, would have been exposed (*27*,*28*). So in effect in this study only the aerosol route of infection was blocked, and this step alone achieved near complete protection.

The converse occurrence, blocking only the large droplet and fomites routes in natural infections, can be inferred from the studies on the use of zanamivir for prophylaxis described previously. In experimental settings, intranasally applied zanamivir was protective against an experimental challenge with influenza by intranasal drops (*21*). However, in studies on prophylaxis of natural disease, intranasal zanamivir was not protective (*22*), which leads to the conclusion that natural infection can occur efficiently by a route other than large droplets or fomites. As noted above, inhaled zanamivir was significantly protective (*22*,*23*).

Discussion and Implications for Infection Control during Influenza A (H5) Pandemic

In principle, influenza viruses can be transmitted by 3 routes: aerosols, large droplets, and direct contact with secretions (or with fomites). These 3 routes are not mutually exclusive and, as noted above, may be difficult to disentangle in natural infections.

For the purpose of deciding on the use of N95 respirators in a pandemic, showing that aerosol transmission occurs at appreciable rates is sufficient. Evidence supporting aerosol transmission, reviewed above, appears compelling. Despite the evidence cited in support of aerosol transmission, many guidelines or review articles nevertheless routinely state that "large droplets transmission is thought to be the main mode of influenza transmission" (or similar statements) without providing supporting evidence from either previously published studies or empirical findings. Despite extensive searches, I have not found a study that proves the notion that large-droplets transmission is predominant and that aerosol transmission is negligible (or nonexistent). Reports on many outbreaks suggest that influenza aerosols are rapidly diluted because long-range infections occur most spectacularly in situations of crowding and poor ventilation (*25*,*26*). However, even if long-range infections do not readily occur when sufficient ventilation exists, this does not rule out the presence at closer range of infectious particles in the micron or submicron range, against which surgical masks would offer little protection (*29*,*30*). Many infection control practitioners have argued that the introduction of large-droplets precautions in institutions has proven sufficient to interrupt influenza outbreaks and therefore that aerosol transmission appears negligible. This evidence is, unfortunately, inconclusive because of several confounding or mitigating

factors. First, unless precise laboratory diagnosis is obtained, respiratory syncytial virus outbreaks can be mistaken for influenza outbreaks (*9*), which would artificially increase the perceived "effectiveness" of large-droplets precautions against influenza. Second, serologic studies are often not conducted, and therefore asymptomatic infections are not documented (among healthcare workers a large fraction of influenza infections are asymptomatic or mistaken for another disease [*31*]). Third, since we are in an interpandemic period and the viruses currently circulating have been drifting from related strains for decades, we all have partial immunity against these viruses, immunity that is further boosted in vaccinated healthcare workers. It has even been argued that after several decades of circulation the current human influenza viruses are undergoing gradual attenuation (*32*). Finally, surgical masks (used in large-droplets precautions) do not offer reliable protection against aerosols, but they nevertheless have a partially protective effect, which further confuses the issue (*29*,*30*).

In contrast, the situation with a pandemic strain of influenza A (H5) would become only too clear because no one would have any degree of immunity against such a virus, vaccines would not be available for months, and these viruses would likely be highly virulent. Even though efficient human-to-human transmission of the A (H5N1) virus has not yet been observed (by any mode), transmission of influenza A (H5N1) by aerosols from geese to quails has been demonstrated in the laboratory (*33*). Thus, even in the current incarnation of A (H5N1), infection by the virus can generate aerosols that are infectious for highly susceptible hosts. As far as we know, 1 of the main blocks to efficient human-to-human transmission of influenza A (H5N1) is the virus's current preference for specific sialic acid receptors. The current strains still prefer α-2,3–linked sialic acids, which is typical of avian influenza viruses, whereas human influenza viruses bind preferentially to α-2,6–linked sialic acids (*34*–*36*). In all likelihood, 1 of the mutations required for influenza A (H5N1) to give rise to a pandemic strain would be to change its receptor affinity to favor the α-2,6–linked sialic acids. For the influenza A (H1N1) pandemic strain of 1918, this change required only 1 or 2 amino acid substitutions (*36*). Once a highly transmissible strain of influenza A (H5) has arisen, it will likely spread in part by aerosols, like other human influenza viruses.

Recent studies have shown that whereas epithelial cells in the human respiratory tract express predominantly the α-2,6 sialic acid receptor, cells expressing the α-2,3 receptor were detected only occasionally in the upper respiratory tract; however, measurable expression of α-2,3–linked sialic acid receptors was found in some cells in the alveolar epithelium and at the junction of alveolus and terminal bronchiole (*35*). Binding of influenza A (H5N1) virus can be demonstrated in human tissue sections from the respiratory tract in a distribution corresponding to that of the α-2,3 receptors in the respiratory tract (*34*,*35*). This pattern of virus binding correlates well with autopsy findings, which show extensive alveolar damage (*34*,*37*), and also correlates well with the observation that recovery of the A (H5N1) virus is much more difficult from nasal swabs than from throat swabs (*37*). Thus, in the respiratory system the current strains of A (H5N1) appear to infect mostly (perhaps exclusively) the lower respiratory tract. If that is indeed the case, it in turn suggests that human cases of avian influenza were acquired by exposure to an aerosol, since large droplets would not have delivered the virus to the lower respiratory tract. (Another hypothesis might be gastrointestinal infection, followed by viremia and dissemination, but not all patients have gastrointestinal symptoms [*37*]). Given the strong evidence for aerosol transmission of influenza viruses in general, and the high lethality of the current strains of avian influenza A (H5N1) (*37*), recommending the use of N95 respirators, not surgical masks, as part of the protective equipment seems rational.

Several infection control guidelines for influenza have recently been published, some specifically aimed at the current strains of A (H5N1), others as part of more comprehensive pandemic plans that address the emergence not only of a pandemic form of A (H5) but also of other types of pandemic influenza viruses. Even though to date human-to-human transmission of A (H5N1) remains very inefficient, the high lethality of the infection and potential for mutations call for prudence. The use of N95 respirators is included in the 2004 recommendations of the Centers for Disease Control and Prevention for healthcare workers who treat patients with known or suspected avian influenza (*38*). The World Health Organization's current (April 2006) guidelines for avian influenza recommend the use of airborne precautions when possible, including the use of N95 respirators when entering patients' rooms (*39*).

Currently, several pandemic plans differ considerably in their recommendations for infection control precautions and PPE. The current version of the Canadian pandemic plan recommends surgical masks only, disregarding data that support the aerosol transmission of influenza (*4*). The US pandemic plans (*5*) and the British plans, from both the National Health Service (available from http:// www.dh.gov.uk/PublicationsAndStatistics/Publications/PublicationsPolicyAndGuidance/PublicationsPolicyAndGuidanceArticle/fs/en?CONTENT_ID=4121735&chk=Z6kjQY) and the Health Protection Agency (http://www.hpa.org.uk/infections/topics_az/influenza/pandemic/pdfs/HPAPandemicplan.pdf), acknowledge the contribution of aerosols in influenza but curiously recommend surgical masks for routine care; the use of N95 respirators is reserved for protection during "aerosolizing procedures"

(*5*,*40*). These recommendations fail to recognize that infectious aerosols will also be generated by coughing and sneezing. The Australian Management Plan for Pandemic Influenza (June 2005) recommends N95 respirators for healthcare workers (http://www.health.gov.au/internet/wcms/Publishing.nsf/Content/phd-pandemic-plan.htm), and in France, the Plan gouvernemental de prévention et de lutte <<Pandémie grippale>>(January 2006) recommends FFP2 respirators (equivalent to N95 respirators) (http://www.splf.org/s/IMG/pdf/plan-grip-janvier06.pdf). Given the scientific evidence that supports the occurrence of aerosol transmission of influenza, carefully reexamining current recommendations for PPE equipment would appear necessary.

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Susceptibility of North American Ducks and Gulls to H5N1 Highly Pathogenic Avian Influenza Viruses

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Since 2002, H5N1 highly pathogenic avian influenza (HPAI) viruses have been associated with deaths in numerous wild avian species throughout Eurasia. We assessed the clinical response and extent and duration of viral shedding in 5 species of North American ducks and laughing gulls (*Larus atricilla*) after intranasal challenge with 2 Asian H5N1 HPAI viruses. Birds were challenged at ≈10 to 16 weeks of age, consistent with temporal peaks in virus prevalence and fall migration. All species were infected, but only wood ducks (*Aix sponsa*) and laughing gulls exhibited illness or died. Viral titers were higher in oropharyngeal swabs than in cloacal swabs. Duration of viral shedding (1–10 days) increased with severity of clinical disease. Both the hemagglutination-inhibition (HI) and agar gel precipitin (AGP) tests were able to detect postinoculation antibodies in surviving wood ducks and laughing gulls; the HI test was more sensitive than the AGP in the remaining 4 species.

Free-living birds in the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shore birds) have traditionally been considered the natural reservoirs for avian influenza viruses (AIVs) (*1*,*2*). However, before 2005, no evidence showed that highly pathogenic avian influenza (HPAI) viruses were maintained in wild bird populations. Rather, HPAI viruses evolved independent of wildlife reservoirs when wild-type AIVs were introduced and adapted to domestic poultry populations (*3*). One exception occurred in 1961 when a high proportion of deaths in common terns (*Sterna hirundo*) in South Africa was attributed to an H5N3 HPAI virus without evidence of prior infection in domestic poultry (*4*). However, this tern epizootic was limited, and the virus did not become endemic in any wild bird population.

In 2002, a substantial number of deaths associated with H5N1 HPAI virus infection were reported in captive ducks, geese, and flamingos housed within 2 waterfowl parks in Hong Kong Special Administrative Region, People's Republic of China (*5*). Free-living gray herons (*Ardea cinerea*) and black-headed gulls (*Larus ridbundus*) also died during these outbreaks. Since 2002, sporadic deaths in wild birds, associated with H5N1 HPAI, have continued (*6*). Beginning in spring 2005, H5N1 HPAI outbreaks involving large numbers of wild birds were reported, and the subsequent spread of these viruses to Europe and Africa suggests that migratory birds may have been responsible for the long-range movement of these viruses. However, which wild avian species are important in H5N1 HPAI movement and whether these viruses will be established in free-living avian populations is unknown. The goal of this study was to determine the susceptibility of critical species of North American waterfowl to 2 H5N1 HPAI viruses and the potential impact of these species on the epidemiology of the viruses in North America.

Material and Methods

Animals

Five species of indigenous North American ducks were used in this study: mallard (*Anas platyrhynchos*), northern pintail (*Anas acuta*), blue-winged teal (*Anas crecca*), redhead (*Aythya americana*), and wood duck (*Aix sponsa*). Species were selected to represent the diverse habitat and behavior of ducks in North America and included important AIV reservoirs (mallard), long-distant migrants (northern pintail and blue-winged teal), diving ducks (redhead), and birds that breed in both northern and southern

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areas of the United States (wood duck). All ducks used in this study were captive-bred and acquired at 10 to 16 weeks of age (Howell's Exotic Waterfowl, Muldrow, OK, USA). This age is consistent with premigration staging in the late summer or early fall when AIV prevalence peaks in wild waterfowl (*7*). Both male and female ducks were included in each species and were approximately equally represented.

Wild-caught gulls used in this investigation were acquired through the Southeastern Cooperative Wildlife Disease Study, University of Georgia (UGA), under federal permit. Nestling laughing gulls (*Larus atricilla*) were hand-caught in McIntosh County, Georgia, by personnel from the Georgia Department of Natural Resources and maintained at the College of Veterinary Medicine, UGA. At 12 weeks of age the gulls were transported to biosafety level 3–agriculture (BSL-3-Ag) facilities at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service, United States Department of Agriculture (USDA).

All birds used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (*8*) and under an animal use protocol approved by the Institutional Animal Care and Use Committee at both SEPRL and UGA. All experiments were performed in the USDA-certified BSL-3-Ag facility at SEPRL (*9*).

Viruses

Two viruses were used in this study: A/Whooper Swan/Mongolia/244/05 (H5N1) (Mongolia/05) and A/Duck Meat/Anyang/01 (H5N1) (Anyang/01). The Mongolia/05 isolate was obtained from a dead whooper swan (*Cygnus cygnus*) and was chosen because of its known lethality in wild waterfowl. The Anyang/01 isolate was chosen on the basis of results from previous experimental infections of Pekin white ducks (*Anas platyrhyncos*), which did not result in illness or death (*10*).

Individual stocks of both AIVs used in this study were produced by second passage in 9-day-old embryonated chicken eggs. Allantoic fluid from the inoculated eggs was diluted in brain-heart infusion (BHI) medium to yield a final titer of 10^6 embryo 50% infectious doses (EID_{50}) per 0.1 mL (single bird inoculum). A sham inoculum was prepared by diluting sterile allantoic fluid 1:30 in BHI.

Experimental Design

Preinoculation serum was collected from each bird to confirm they were serologically naïve to influenza A viral antigens by agar gel precipitin test (AGP) and to H5 influenza by specific hemagglutination inhibition (HI) testing by using standard procedures (*11*). In addition, oropharyngeal and cloacal swabs were collected before inoculation to confirm an AIV-free status. The 5 species of ducks and laughing gulls were each separated into a control group and 2 virus-inoculated groups (Mongolia/05 and Anyang/01), each consisting of 3 birds. Ducks and gulls were inoculated intranasally with a 0.1-mL volume of the designated virus solution or sham-inoculum. All birds were monitored daily for illness or death. Due to the lack of illness exhibited by most ducks, experiments with these species were extended to 20 days postinoculation (DPI) to allow adequate time for seroconversion. Cloacal and oropharyngeal swabs were collected in BHI medium with antimicrobial drugs (100 μg/mL gentamicin, 100 units/mL penicillin, and 5 μg/mL amphotericin B) from all birds at 1, 2, 3, 4, 5, 7, 10, and 14 DPI. Oropharyngeal and cloacal swabs were also collected on 20 DPI from the 5 species of ducks. At 14 DPI (gulls) or 20 DPI (ducks), serum was collected from the surviving birds for serologic testing with HI and AGP, and the birds were humanely killed with intravenous sodium pentobarbital (100 mg/kg bodyweight). Serum was not collected from birds that died during the course of the study (that were not killed at the end of the study). Necropsies were performed on all birds, and routine tissues were collected for histopathologic and immunohistochemical evaluation. In addition, portions of heart, breast muscle, kidney, lung, and brain, and oropharyngeal and cloacal swabs were collected and stored in BHI medium with antimicrobial drugs for virus isolation.

Histopathologic and Immunohistochemical Analysis

Tissues samples collected at necropsy were preserved in 10% neutral buffered formalin. After fixation, the tissues were routinely processed and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin. Duplicate sections were immunohistochemically stained by using a mouse-derived monoclonal antibody (P13C11) specific for type A influenza virus nucleoprotein antigen as the primary antibody (SEPRL, Athens, GA, USA). Procedures used to perform the immunohistochemical testing followed those previously described (*12*). Fast red was used as the substrate chromagen, and slides were counterstained with hematoxylin. Demonstration of viral antigen was based on chromagen deposition in the nucleus, with or without chromagen deposition in the cytoplasm.

Virus Isolation

Oropharyngeal and cloacal swabs and tissue samples collected at necropsy were stored at −70°C until virus isolations and titrations were performed. Isolation of virus from swabs and tissues was performed by using embryonated chicken eggs (*11*). Positive samples were titrated by

determining the EID_{50}. The minimal detectable titer was $10^{0.97}$ EID_{50}/mL from swabs and $10^{1.97}$ EID_{50}/g from tissues.

Serologic Assays

AGP and HI tests were performed on the pre- and postinoculation serum by using standard procedures (*11*). The HI tests were performed by using a 0.5% suspension of chicken erythrocytes in phosphate-buffered saline.

Phylogenetic Analysis

In addition to the 2 H5N1 viruses used in this study, A/chicken/Hong Kong/220/97 (H5N1) (Hong Kong/97) was included in the phylogenetic analysis because it is the only other H5N1 HPAI virus evaluated in multiple avian species by experimental inoculation (*13*). Sequence comparisons of these 3 viruses were conducted with the MegAlign program by using the ClustalV alignment algorithm (DNASTAR, Madison, WI, USA), and phylogenetic relationships were estimated by the method of maximum parsimony (PAUP software, version 4.0b10; Sinauer Associates, Inc, Sunderland, MA, USA) by using a bootstrap resampling method with a heuristic search algorithm. Pairwise sequence comparisons were done within the MegAlign program.

Results

Morbidity and Mortality Data

Morbidity and mortality data are summarized in Table 1. Wood ducks were the only species of duck to exhibit illness or death after inoculation with either of the HPAI viruses. Severe clinical disease developed in 2 Mongolia/05-inoculated wood ducks, characterized by cloudy eyes, ruffled feathers, rhythmic dilation and constriction of the pupils, severe weakness, incoordination, tremors, and seizures (Figure 1). One of these ducks died at 7 DPI and the other was humanely killed at 8 DPI because of its moribund condition. Two Anyang/01-inoculated wood ducks became ill with clinical signs similar to those described for the Mongolia/05 virus group. One of these ducks died, and the other slowly recovered over 7 days until it exhibited no clinical symptoms. One wood duck in each viral group remained clinically normal for the entire trial. Clinical signs were not observed in the remaining duck species.

All 3 Mongolia/05-inoculated laughing gulls exhibited severe clinical signs consisting of cloudy eyes, ruffled feathers, weakness, and incoordination, torticollis, or both. Two of these gulls died. The remaining gull clinically improved and stabilized over 6 days, but retained a head-tilt for the remainder of the trial. Severe clinical signs developed in all Anyang/01-inoculated gulls, similar to those seen in Mongolia/05-inoculated gulls. The disease progressed to death in 2 of these gulls. The remaining gull exhibited clinical signs for 8 days but gradually recovered until it showed no clinical symptoms.

Pathologic Features

Viral-induced lesions were found only in the wood ducks and laughing gulls that exhibited clinical signs. Lesions were mild in birds that recovered but were severe and widespread in birds that died or were humanely killed due to severe illness. For each species, the severity and

Table 1. Morbidity, mortality, and virus isolation data from 5 species of ducks and laughing gulls* intranasally inoculated with 2 different H5N1 HPAI viruses†

			Virus isolation (oral swab)			Virus isolation (cloacal swab)		
Virus/Host	No. sick/ total (‡)	No. dead/ total (§)	Prevalence, no positive/total	Duration, d	AMT¶ (log_{10} EID_{50}/mL)	Prevalence, no. positive/total	Duration, d	AMT (log_{10} EID_{50}/mL)
Mongolia/05								
BWT	0/3	0/3	3/3	2	3.8	1/3	1	1.0
RD	0/3	0/3	3/3	1–4	2.8	2/3	1	1.2
WD	2/3 (5)	2/3 (7,8)	3/3	4–6	4.6	2/3	2,3	3.8
NP	0/3	0/3	3/3	1–2	1.5	1/3	1	1.0
LG	3/3 (2–5)	2/3 (7,8)	3/3	7–8	4.2	3/3	4–7	2.6
Anyang/01								
BWT	0/3	0/3	2/3	1,2	2.0	0/3	–	–
RD	0/3	0/3	2/3	4	4.0	0/3	–	–
WD	2/3 (6)	1/3 (8)	3/3	7	5.0	2/3	4,5	2.8
MD	0/3	0/3	3/3	1–2	2.1	1/3	1	1.0
NP	0/3	0/3	2/3	1,4	1.1	0/3	–	–
LG	3/3 (3–5)	2/3 (6–10)	3/3	6–10	5.0	3/3	3–6	2.0

*Intranasally sham-inoculated control birds for each avian species lacked clinical, serologic, virologic, and pathologic evidence of avian influenza virus infection.
†HPAI, highly pathogenic avian influenza; BWT, blue-winged teal; RD, redhead; WD, wood duck; MD, mallard; NP, northern pintail, LG, laughing gull; Mongolia/05, A/Whooper Swan/Mongolia/244/05; Anyang/01, A/Duck Meat/Anyang/01.
‡No. in parentheses indicates the first day postinoculation that clinical disease was apparent.
§No. in parentheses indicates day of death.
¶Average maximum titer (AMT) is the average peak titer for birds that shed virus (log_{10} 50% embryo infective dose/mL).

Figure 1. A female wood duck with severe neurologic clinical signs of disease after intranasal inoculation with an Asian strain of highly pathogenic avian influenza H5N1 virus.

distribution of lesions were the same for both H5N1 viruses, with the following exception described below.

Gross lesions were not present in any of the recovered birds. Wood ducks that died had multiple petechial hemorrhages in the pancreas, whereas laughing gulls had more widely distributed petechial hemorrhages in the ventriculus, apex of the heart, cerebrum, and pancreas.

On histopathologic examination, wood ducks that died had severe, diffuse neuronal necrosis in the cerebrum (Figure 2A) and, less commonly, in the cerebellum. Other common lesions included necrotizing pancreatitis (Figure 2D) and adrenalitis (Figure 2C) and multifocal myocardial necrosis. Myocardial necrosis was only observed in wood ducks inoculated with Mongolia/05 and not with Anyang/01. Necrotizing pancreatitis and cerebral neuronal necrosis were the most common lesions in gulls that died during the study. Necrotizing adrenalitis was also observed in gulls that died but was less common and milder than the changes in the pancreas and cerebrum. Microscopic lesions in wood ducks and laughing gulls that recovered were less severe than in those that died. In both species, the most common lesions in recovered birds were lymphoplasmacytic perivascular encephalitis and heterophilic pancreatitis.

Wood ducks that died during the study had viral antigen in numerous organs, including the brain (Figure 2B), adrenal glands, testicles, kidneys, liver, small intestines, heart, skeletal muscles, pancreas, and air sacs. Viral antigen was most frequently found in cardiac myocytes, parasympathetic ganglia in the submucosal and muscular plexus of the small intestines, and numerous cell types in the brain, including glial cells, ependymal cells, endothelial cells, neurons, and gitter cells. Viral antigen was also detected in the pancreatic acinar cells and cortical and medullary cells of the adrenal gland, although less often than the aforementioned sites. Minimal amounts of viral antigen were detected in the kidney and testis in 1 and 2 wood ducks that died, respectively. The 1 wood duck that recovered had a scant amount of viral antigen in the cerebellar neurons. Laughing gulls that died during the study had viral antigen most frequently detected in the neurons, endothelial cells, glial cells, and ependymal cells in the brain, pancreatic acinar cells, and cortical and medullary cells of the adrenal glands. Laughing gulls that died also had minimal amounts of viral antigen present in other organs including the heart, lungs, air sacs, thymus, kidneys, small intestines, and eyes. Laughing gulls that recovered contained small amounts of viral antigen in the pancreatic acinar cells and cerebral and cerebellar neurons.

Virus Isolation and Serologic Testing

The virus isolation results are summarized in Tables 1 and 2. Viral titers were higher in oropharyngeal swabs than in cloacal swabs in all species and with both H5N1 viruses. Viral titers on cloacal swabs were low, except from birds that died of AIV infection. Oropharyngeal swabs from all species collected at 1 and 2 DPI were positive on virus isolation. Wood ducks and laughing gulls had higher viral titers on oropharyngeal and cloacal swabs and shed virus longer than any of the other species. Virus was isolated from numerous organs in the wood ducks and laughing gulls that died.

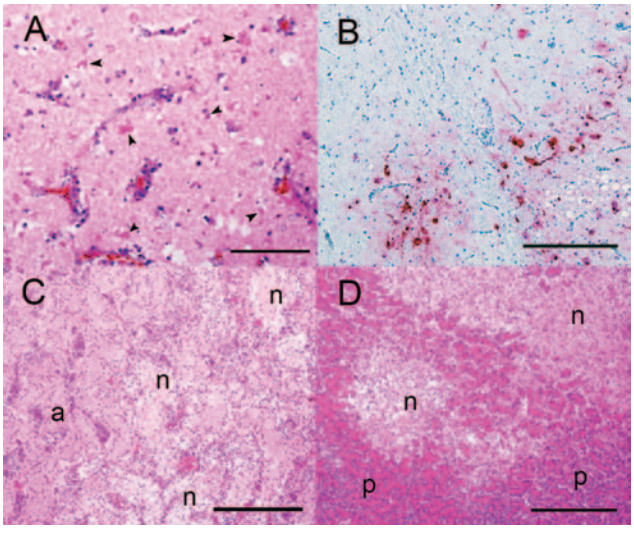

Figure 2. Photomicrographs of visceral organs from a wood duck that died after intranasal inoculation with a highly pathogenic avian influenza H5N1 virus. A) Brain with severe, multifocal to coalescing neuronal necrosis. Note the numerous necrotic neurons (arrowheads). Hematoxylin and eosin (HE) stain; bar =100 μm. B) Brain. Note the viral antigen (red) detected in the nucleus of several neurons. The unaffected brain tissue is blue. Immunohistochemical stain with hematoxylin counterstain; bar = 200 μm. C) Adrenal gland with necrotizing adrenalitis. Note the multiple foci of necrosis (n) surrounded by normal adrenal parenchyma (a). HE stain; bar = 200 μm. D) Pancreas with necrotizing pancreatitis. Note the 2 well-demarcated areas of necrosis (n) within the normal pancreatic tissue (p).

Table 2. Mean viral titer for tissues from wood ducks and laughing gulls* that died after inoculation with 2 different HPAI H5N1 viruses†

Host virus	Brain	Heart	Lung	Skeletal muscle	Kidney
WD-Anyang	3.7‡	3.5	5.1	ND§	2.9
WD-Mongolia	6.6	2.7	7.1	2.5	6.7
LG-Anyang	4.8	4.7	5.2	2.5	4.2
LG-Mongolia	6.3	2.5	3.3	4.2	2.5

*No virus was isolated from the internal organs of the other 4 avian species inoculated with H5N1 viruses and all of the sham-inoculated control birds.
†HPAI, highly pathogenic avian influenza; WD, wood duck; LG, laughing gull; Anyang, A/Duck Meat/Anyang/01; Mongolia, A/Whooper Swan/Mongolia/244/05.
‡$\log_{10}$ mean 50% embryo infectious dose per gram ($\log_{10}EID_{50}/g$).
§Not detected.

Serologic testing results are summarized in Table 3. Both the AGP and HI tests detected postinoculation antibodies in all surviving wood ducks and laughing gulls. However, the effectiveness of these tests in the remaining duck species was variable and dependent on host species and inoculated virus. The HI test detected postinoculation antibodies in multiple avian species that had little serologic response or none as determined by the AGP test (Anyang/01-inoculated mallards, redheads, and northern pintails and Mongolia/05-inoculated mallards). Both serologic tests were least effective in northern pintails and mallards.

Molecular Biology

In comparing the 3 viruses (Hong Kong/97, Anyang/01, and Mongolia/05) genetically, the hemagglutinin genes are all clearly in the Goose/Guandong/96 lineage. At the amino acid (aa) level they vary by 3.5%–4.8%. They all have the HA cleavage compatible with an HPAI phenotype. The cleavage site is the same for Hong Kong/97 and Anyang/01, but the Mongolia/05 virus has 2 aa changes at the cleavage site. Phylogenetically, Hong Kong/97 and the Anyang/01 are in or close to clade 3, and the Mongolia/05 strain is in clade 2 (*14*). The Mongolia/05 strain appears to be a representative isolate from the wild bird viruses that have been reported in Asia, Europe, and Africa.

Comparison of the other 7 gene segments demonstrates evidence of reassortment. The viruses from the 1997 outbreak in Hong Kong have a unique subtype-1 neuraminidase gene compared with any of the other H5N1 viruses. The Anyang/01 and Mongolia/05 N1 genes are from the same lineage, and both have an identical 20 aa stalk deletion. For the other 6 internal genes, the Anyang/01 and Mongolia/05 viruses in general were more closely related to each other than to the Hong Kong/97 virus. Except for the H5 gene, the Hong Kong/97 and viruses isolated in Hong Kong in the same year appear to be a unique constellation of genes that has not been seen again. Although the Anyang/01 and Mongolia/05 viruses were more closely related, the internal genes are most likely the result of reassortment with other influenza viruses and not the result of progressive sequence in a single lineage of viral genes.

Discussion

Data from this study indicate that wood ducks and laughing gulls are highly susceptible to infection with H5N1 HPAI viruses as evidenced by widespread microscopic lesions, prolonged and highly concentrated viral shedding, and seroconversion. In addition, these species are likely to exhibit clinical disease or death associated with H5N1 virus infection. In a previous study, 2- to 3-week-old laughing gulls inoculated with A/chicken/Hong Kong/220/97 (H5N1) and A/tern/South Africa/61 (H5N3)

Table 3. Serology data from 5 duck species and laughing gulls inoculated with 2 different H5N1 HPAI viruses*

	AGP serology		HI serology	
Virus/host	Prechallenge, no. positive/total	Postchallenge, no. positive/total	Prechallenge, no. positive/total (GMT†)	Postchallenge, no. positive/total (GMT†)
Mongolia/05				
BWT	0/3	3/3	0/3	3/3 (13)
RD	0/3	3/3	0/3	3/3 (26)
WD	0/3	1/1	1/3 (8)	1/1 (128)
MD	0/3	0/3	0/3	1/3 (64)
NP	0/3	0/3	0/3	0/3
LG	0/3	1/1	1/3 (8)	1/1 (64)
Anyang/01				
BWT	0/3	0/3	0/3	3/3 (10)
RD	0/3	1/3	0/3	3/3 (20)
WD	0/3	2/2	0/3	2/2 (64)
MD	0/3	0/3	0/3	2/3 (16)
NP	0/3	0/3	0/3	2/3 (8)
LG	0/3	1/1	0/3	1/1 (32)

*HPAI, highly pathogenic avian influenza virus; AGP, agar gel precipitin; HI, hemagglutination inhibition; BWT, blue-winged teal; RD, redhead; WD, wood duck; MD, mallard; NP, northern pintail; LG, laughing gull; Anyang/01, A/Duck Meat/Anyang/01; Mongolia/05, A/Whooper Swan/Mongolia/244/05.
†GMT, geometric mean titer.

did not exhibit illness or death (*15*). Viral replication in these birds was minimal and restricted to the respiratory tract. Since 2002, some emergent H5N1 viruses have exhibited unique characteristics, including lethality for waterfowl (*16*). Consistent with previous studies of ducks (*17*), the more recent isolates of H5N1 viruses used in our study caused a high proportion of illness and death in gulls, whereas the earlier H5N1 isolate, mentioned above, did not. To our knowledge, this is the first experimental inoculation of wood ducks with any HPAI viruses. Our results are consistent with field data that also indicate that wood ducks are highly susceptible to H5N1 HPAI viruses. In an investigation of H5N1 virus outbreaks in 2 waterfowl parks in Hong Kong, 18 of the 26 wood ducks on the lakes died (*5*). Of the wood ducks that died, 16 were positive for H5N1 virus by culture.

Traditionally, ducks asymptomatically shed high concentrations of wild-type AIVs in their feces (*18*). In this paradigm, ducks can transmit AIV over great distances as they migrate, and these viruses can remain infectious for prolonged periods of time in water (*18*,*19*). This fecal-oral mechanism is efficient at maintaining these viruses within duck populations and also transmitting AIVs from wild ducks to domestic poultry. Predominant oropharyngeal shedding has been consistently demonstrated with these H5N1 HPAI viruses (*20*), as it was in our study, and what impact this shedding pattern may have on environmental contamination, persistence in aquatic habitats, and transmission between birds (both wild and domestic) is unknown.

An efficient surveillance system is central to any preparedness program aimed to detect H5N1 in North America. Our data indicate that wood ducks and laughing gulls would be sensitive indicators of the presence of H5N1 circulating in wild birds. Wild avian species have previously been included in monitoring programs for other infectious diseases, for example, crow deaths for detection of West Nile virus (*21*). Other wild avian species in North America would also likely serve as sensitive indicators, but predicting which species is not possible without experimental inoculations or consistent morbidity and mortality data from outbreaks.

In relation to wood ducks and laughing gulls, the remaining 4 duck species were much less susceptible to H5N1 HPAI virus infection and were refractory to disease. Although these species may possibly contribute to viral transmission in wild avian populations, their role in the spread or maintenance of H5N1 HPAI virus is probably minimal. However, our experimental results are based on small sample sizes (n = 3) that are inadequate to fully evaluate potential individual bird variation in response to H5N1 challenge.

Illness, deaths, and viral shedding were less in our study than what has previously been reported for experimental inoculation of ducks with H5N1 HPAI virus. Possible explanations for the reduced pathogenicity include the age of birds used in the study and the variability between different H5N1 HPAI viruses. An age-dependent reduction in lethality was present between 2- and 4-week-old ducks inoculated intranasally with H5N1 HPAI virus (*22*). Similarly, previous experimental infections of mallards 2- to 6-weeks old with H5N1 resulted in a higher proportion of deaths than we observed with 10- to 16-week-old ducks (*17*,*20*,*23*). Experimental infections of very young ducks may overestimate the susceptibility of a species and the results may be incongruent with morbidity and mortality field data. The reduced pathogenicity and infectivity could also be characteristic for the specific H5N1 viruses used in this study.

One wood duck and 1 laughing gull reacted positively for preinoculation antibodies to AIV by the HI test. However, both of these birds were positive at the lowest detectable limit of this test, and these results may have been false-positive due to nonspecific hemagglutination. The wood duck did not become sick after inoculation with the Mongolia/05 isolate. The laughing gull did become ill after inoculation with the Anyang/01 virus, but completely recovered. If these serologic results are true positives, it is possible that the low antibody titers provided some immunologic resistance for these birds.

Serologic techniques commonly used in domestic poultry have limitations in ducks. The results of this study suggest variation between wild duck species in the ability of the AGP and HI tests to detect antibodies to type A influenza virus and H5 AIVs, respectively. Although the HI test was more sensitive than the AGP in detecting antibodies in our study, both serologic tests did not detect antibodies to AIV in some postinoculation serum samples from experimentally infected ducks. Furthermore, when duck erythrocytes were used in place of chicken erythrocytes for the HI test, antibodies were not detected in some of the duck samples (J. Brown, unpub.data). Surveillance systems that rely on these serologic techniques to detect H5N1 HPAI virus in ducks may substantially underestimate the prevalence of virus. In addition, false-positive results are possible if HI testing is used in H5N1 surveillance because positive results for H5 AIVs indicate previous infection with H5N1 HPAI virus or any other H5 wild-type AIV. Further information is necessary to evaluate the efficacy of these serologic assays in other wild avian species to allow correct interpretation of serologic field data.

The genetic sequence information indicated that all 3 evaluated H5N1 HPAI viruses were genetically distinct from each other, although the Anyang/01 and Mongolia/05

viruses were overall more closely related to each other than to Hong Kong/97. The only gene segment that all 3 viruses shared as part of a single viral lineage was the hemagglutinin gene. All 3 viruses had cleavage sites compatible with HPAI viruses, and experimental inoculation showed them to be extremely virulent in chickens (*10*,*24*, D. Swayne, unpub. data). Because of the large sequence differences, which genetic changes account for the virulence or host specificity differences cannot be identified. Reverse genetics has shown that single amino acid differences can greatly affect virulence, such as the change of glutamine to lysine at position 627 in the PB2 gene. This single difference can greatly increase the virulence of Hong Kong/97 viruses in mice (*25*).

The results of this study indicate that there is significant species-related variation in susceptibility, clinical disease, and antibody response to H5N1 virus infection in wild birds. Predicting this susceptibility beyond the species examined in this study is not possible. Wood ducks and laughing gulls were highly susceptible to H5N1 HPAI viruses with substantial illness and death. If H5N1 were introduced into North America, these species may serve as effective indicator species in a surveillance program.

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Targeted Social Distancing Design for Pandemic Influenza

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Targeted social distancing to mitigate pandemic influenza can be designed through simulation of influenza's spread within local community social contact networks. We demonstrate this design for a stylized community representative of a small town in the United States. The critical importance of children and teenagers in transmission of influenza is first identified and targeted. For influenza as infectious as 1957-58 Asian flu (≈50% infected), closing schools and keeping children and teenagers at home reduced the attack rate by >90%. For more infectious strains, or transmission that is less focused on the young, adults and the work environment must also be targeted. Tailored to specific communities across the world, such design would yield local defenses against a highly virulent strain in the absence of vaccine and antiviral drugs.

At the start of an influenza pandemic, effective vaccine and antiviral drugs may not be available to the general population (*1*,*2*). If the accompanying illness and death rates of the virus strain are high, how might a community respond to protect itself? Closing roads, restricting travel, and community-level quarantine will enter discussions (*3*,*4*). However, within a community, influenza spreads from person to person through the social contact network. Therefore, understanding and strategically controlling this network during a period of pandemic is critical.

We describe how social contact network–focused mitigation can be designed. At the foundation of the design process is a network-based simulation model for the spread of influenza. We apply this model to a community of 10,000 persons connected within an overlapping, stylized, social network representative of a small US town. After study of the unmitigated transmission of influenza within the community, we change the frequency of contact within targeted groups and build combinations of strategies that can contain the epidemic. Finally, we show how infectivity of the strain and underlying structure of the infectious contact network influence the design of social distancing strategies. In the absence of vaccine and antiviral drugs, design for specific communities would defend against highly virulent influenza.

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Methods

The design process first creates an explicit social contact network in which persons are linked to others in a community. Spread of influenza within the network is then simulated by imposing behavioral rules for persons, their links, and the disease. These rules are modified to implement targeted mitigation strategies within the community, the effectiveness of which is evaluated (*5*).

Contact Network

A network is created by specifying groups of given sizes (or range of sizes) within which persons of specified ages interact (e.g., school classes, households, clubs). The average number of links per person within the group is also specified because cliques form or are imposed (e.g., seating in a classroom). This number is used to construct a within-group network that can take various forms. We used fully connected, random, or ring networks for each group. Random networks are formed by randomly choosing 2 persons within the group and linking them. This process is repeated until the number of links within the group yields the specified average (each person will have a different number of links). The ring is formed by first placing persons next to neighbors and linking them to form a complete circle. Additional links are then made to next nearest neighbors symmetrically around the ring. Finally, links within a group are given an average frequency of contact per day. With this approach, a network can be built from the experience of community members to exhibit the clustered yet small-world characteristics (*6*) and overlapping quality of a structured community (*7*,*8*).

Our network represented a stylized small US town and took advantage of the diverse backgrounds of the authors (1 of whom is a teenager). The population of 10,000 conformed to the 2000 Census (*9*) and consisted of children (<11 years of age, 17.7%), teenagers (12–18 years of age, 11.3%), adults (19–64 years of age, 58.5%), and older adults (≥65 years of age, 12.5%). All persons belonged to multiple groups, each associated with a subnetwork of links that reflected their lives within the community (Figure 1, Table 1). Households were composed of families (adults with children or teenagers), adults, or older adults. The age-class makeup and size of households conformed to the 2000 Census (*9*). All persons within each household were linked to each other with mean link contact frequencies of 6/day. Every person also belonged to 1 multiage extended family (or neighborhood) group (mean membership 12.5, mean link contact frequency 1/day).

All children and teenagers attended preschool or school; children attended 1 class/day, while teenagers attended 6 (classes of 20 to 35 children or teenagers). All adults went to work daily, where they interacted with other adults (work group size 10–50), and all older adults attended gatherings with other older adults (gathering size 5–20). For links within school classes, work, and gatherings of older adults, we assumed the simplest subnetwork that imposes local clustering: a ring lattice in which a person is linked to 2 (for children or teenager classes and gatherings of older adults) or 3 (adult work) neighboring persons on each side along the ring. Mean link contact frequencies for children in a class are 6/day. Teenager classes, adult work, and gatherings of older adults have mean link contact frequencies of 1/day.

To represent additional within-age class interactions, such as extracurricular activities, playgrounds, bowling leagues, or friends, persons are randomly linked to an average of 3 other persons of the same age class (mean link contact frequency 1/day). Finally, to emulate a somewhat patterned set of random contacts from commercial transactions and other ventures into public spaces, we impose a random overall network across all age classes with a mean of 25 links/person to yield 1 contact/person/day (mean link contact frequency 0.04/day).

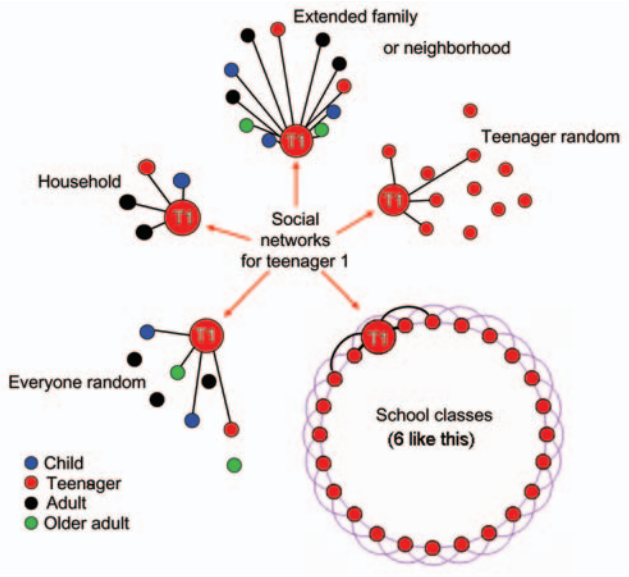

Figure 1. Groups and typical person-to-person links for a model teenager. The teenager (T1) belongs to a household (fully connected network, mean link contact frequency 6/day), an extended family or neighborhood (fully connected network, mean link contact frequency 1/day), and 6 school classes (ring network with connections to 2 other teenagers on each side as shown in black; purple links denote connections of other teenagers within the class; mean link contact frequency 1/day). Two random networks are also imposed, 1 within the age group (teenager random, average of 3 links/teenager, mean link contact frequency of 1/day), and 1 across all age groups (overall random, average of 25 links/person [not all links shown], mean link contact frequency of 0.04/day).

Behavioral Rules

The spread of influenza within the contact network is modeled as a series of 2 classes of events: transition of a person between disease states and person-to-person transmission of influenza. Disease state transitions follow the natural history of influenza (Figure 2). After the latent state, an infected person transitions to an infectious presymptomatic state or an infectious asymptomatic state with probability *pS* or *1 – pS*, respectively. Those with symptoms either stay home with probability *pH*, thus influencing their contacts, or continue to circulate with probability *1 – pH*. Infected asymptomatic persons continue interacting without behavioral changes. Persons who are symptomatic die or become immune with probability *pM* or *1 – pM*, respectively, and asymptomatic persons become immune. Because this final transition does not influence the spread of the disease, we use *pM = 0*.

Person-to-person transmission events are evaluated at the beginning of each period during which a person is infectious. Assuming contact events are statistically independent, a transmission time for each infectious person's links within the contact network is chosen from an exponential distribution with a mean of the link's contact frequency scaled by $I_D \times I_R \times I_A \times S_P \times S_A$, where I_D is the infectivity of the disease, I_R is the relative infectivity of the disease state, S_P is the susceptibility of people to the disease (here taken as 1.0), I_A is the relative infectivity of the person who is transmitting, and S_A is the relative susceptibility of the person receiving. If the transmission time is less than the period during which the person will be in an infectious state (also chosen from an exponential distribution with the prescribed means; Figure 2), transmission is scheduled at

Table 1. Groups, membership, networks, and mean frequencies of contact per link

Group (no. groups in community)	Membership	Average no. links per member	Network type and parameters	Mean frequency of contact per link per day
Households without older adults (2,730)	1–2 adults, 0–4 children, 0–4 teenagers, mean size 3.13	2.13	Fully connected	6
Households with older adults (742)	1–2 older adults, mean size 1.75	0.75	Fully connected	6
Extended families or neighborhoods (800)	0–2 older adults, 0–8 adults, 0–8 teenagers, 0–8 children, mean size 12.5	11.5	Fully connected	1
Child classes (69)	1 class per child, 20–35 children in each	4	Ring network, 2 neighbors on either side	6
Child random (1)	All children	3	Random network link density 3/1,769	1
Teenager classes (264)	6 classes per teenager, 20–35 teenagers in each	4	Ring network, 2 neighbors on either side	1
Teenager random (1)	All teenagers	3	Random network link density of 3/1,129	1
Adult work (351)	1 work group per adult, 10–50 adults in each	6	Ring network, 3 neighbors on either side	1
Adult random (1)	All adults	3	Random network link density of 3/5,849	1
Older adult gathering (156)	1 gathering per person, 5–20 persons in each	4	Ring network, 2 neighbors on either side	1
Older adult random (1)	All older adults	3	Random network link density of 3/1,249	1
Overall random (1)	All age classes	25	Random network link density of 25/9,999	1/25 a day

the chosen time. Otherwise, transmission along that link does not occur during that period. All transmission parameters and contact frequencies may be modified in each of the states, as well as varied among age classes by relative scaling factors such as I_R. In this way, disease representations and mitigation strategies are implemented.

Most influenza-specific parameters used here reflect those of (*10,11*). We approximated normal influenza viral shedding data (*15*) with a time varying infectivity through choice of state periods and relative infectivity scaling factors (Figures 2 and 3). The latent period is a constant (0.75 days) followed by a variable period (mean 0.5 days). The presymptomatic period (mean 0.5 days) has an I_R of 0.25 after which it increased to 1.0 for the first part of the symptomatic period (mean 0.5 days), when viral shedding is maximum and coughing begins. I_R is then reduced to 0.375 for the remainder of the infectious symptomatic period (mean 1 day). For infectious asymptomatic persons, I_R was set at 0.25 for a mean period of 2.0 days, making these persons half as infective as those with symptoms. We chose *pS* as 0.5, *pH* as 0.5 for adults and older adults and *pH* as 0.9 for children and teenagers. When a person is in the symptomatic stay-home state, we reduce the frequency of all nonhousehold connections by 90%. Because children and teenagers have closer contact with others and are less likely to wash hands or control coughs (*16*), they are more infective and susceptible: I_A and S_A are both 1.5 for children, 1.25 for teenagers, and 1.0 for adults and older

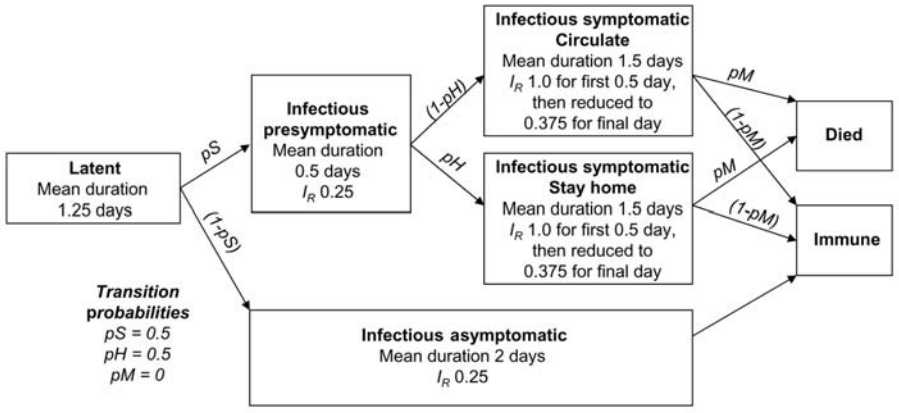

Figure 2. Natural history of influenza in our model. Duration of each state for a given person is chosen from an exponential distribution. State relative infectivity (I_R) and mean state duration were chosen to reflect the infectivity variation of Ferguson et al. (*10,11*) (see Figure 3). Transition probabilities between presymptomatic and postsymptomatic states are also noted. For symptomatic persons who stay at home, link frequencies outside the household are reduced by 90%.

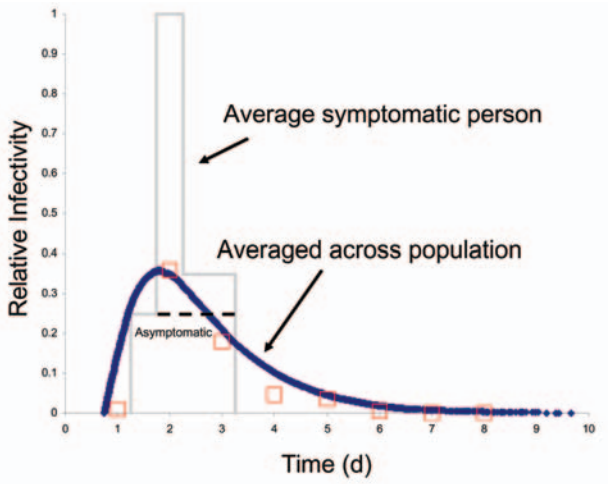

Figure 3. Functional behavior of I_R with time. Although infectivity of an asymptomatic person is constant with time (I_R 0.25), infectivity of a symptomatic person changes from infectious presymptomatic (I_R 0.25) to early infectious symptomatic (I_R 1.0) to late symptomatic (I_R 0.375). A symptomatic person with mean state periods as denoted in Figure 2 is shown in gray (asymptomatic with dashed line). Because state periods are different for each person (given by exponential distributions) and half of the infected persons are asymptomatic, the average population scale I_R in time is smoothed as shown in blue. Both disease state periods and I_R values were chosen to honor the clinically derived natural history of influenza (*12–14*), scaled viral shedding data shown as open red squares (*15*), and the model of Ferguson et al. (*10,11*).

adults. Finally, I_D is adjusted to yield specified attack rates within the community.

Results

We first show the spread of influenza within our unmitigated base case defined with parameters specified above and with I_D chosen to yield an infected attack rate ≈50% to reflect the 1957–58 Asian influenza pandemic (*10*). Unless otherwise noted, we report infected attack rates and refer to them as simply attack rates rather than reporting the illness attack rate which is half of this value (pS = 0.5). We then demonstrate the design of effective local mitigation strategies for the base case that focus on targeted social distancing. Finally, we extend these results to design strategies for more infectious strains and for changes to the underlying infectious contact network that deemphasize the role of children and teenagers.

All simulations are initialized by infecting 10 randomly chosen adults with the assumption that adults are first to be infected through business travel or interaction with visitors from outside the community (*5*). Some of these initial infections instigate others and grow into an epidemic. Results vary across multiple realizations of the community network and random choice of initially infected adults (controlled by random number seed) not all of which yield an epidemic, defined when the number infected is >1% of the population. For every set of parameters, we conducted ≥100 simulations with different random number seeds and collected statistics for all simulations and for only those that result in epidemics (Table 2).

Unmitigated Base Case

The sequence of infected persons can be represented as an expanding network of infectious transmissions (Figure 4). The number of secondary infections produced by an infected person, or branching factor, is easily visualized within the infectious contact network. The average branching factor depends on the person's age class and generation during the epidemic (Figure 5A). The maximum value within the first 10 generations is 2.05 (standard deviation [SD] 0.57) for children, 2.09 (SD 0.72) for teenagers, 1.11 (SD 0.43) for adults, 0.81 (SD 0.47) for older adults, and 1.54 (SD 0.36) for the entire population. Variability (large SD, especially for specific age classes) reflects the heterogeneity inherent within community contact networks of this size (Figure 5B).

The backbone of infectious contact networks is formed primarily of children and teenagers with infectious transmissions mostly in the household, neighborhood, and schools. Infectious transmissions are highest in households without older adults (39%, SD 3%), followed by extended families or neighborhoods (25%, SD 1%), schools (19%, SD 1%), work (7%, SD 2%), combined random groups (9%, SD 2%), and households with older adults (1%, SD 0.1%). On average, 78% (SD 2%) of children and 71% (SD 3%) of teenagers become infected. Adults (attack rate 44% of adults, SD 2%) get influenza mainly from children, teenagers, and other adults within the family. Older adults, who contact children and teenagers only through the extended family or neighborhoods and the random overall network, are relatively isolated (attack rate 23% of older adults, SD 2%).

Children and teenagers compose only 29% of the population yet they are responsible for 59% (SD 4.5%) of infectious contacts, adults for 38% (SD 7.9%), and older adults for 3% (SD 0.6%) (Table 3). Approx-imately half of infectious contacts of either children or teenagers are within the same age class (19%, SD 0.8% and 9%, SD 0.7%, respectively). Adults get influenza from children or teenagers at approximately the same frequency (24%, SD 1.6%) as from other adults (26%, SD 5.9%). Older adults are equally likely to get influenza from children or teenagers as from adults or older adults (2%, SD 0.3%). Transmission to children or teenagers from adults is 10% (SD 1.8%) and nearly none by older adults. These transmission results are supported by recent field studies that show children who go to preschool or school are more likely to contact influenza and their family members are

Table 2. Results for base case and mitigation strategies*

Strategy	Averages for all simulations					Averages for simulations with epidemics				
	No. simulations	Total infected	Total time (d)	Peak infected	Time to peak (d)	No. epidemics	Total infected	Total time (d)	Peak infected	Time to peak (d)
Case 1: Base case pandemic influenza										
Average	1,000	4,908	81	688	35	978	5,018	82	703	36
SD		748	14	121	8		153	11	66	6
Case 2: Schools closed after 10 symptomatic cases, compliance 90%										
Average	100	3,877	113	326	48	99	3,916	114	329	48
SD		468	22	64	13		259	19	56	12
% reduction from base case		21	–40	53	–36		22	–39	53	–34
Case 3: Schools closed after 10 symptomatic cases, nonschool contacts doubled, compliance 90%										
Average	100	5,604	76	850	34	95	5,898	79	894	35
SD		1,293	18	206	9		122	10	72	6
% reduction from base case		–14	6	–24	4		–18	4	–27	2
Case 4: Schools closed after 10 symptomatic cases, children and teenagers kept home, household contacts doubled, compliance 90%										
Average	100	341	60	43	16	93	361	62	45	17
SD		209	25	20	12		203	24	19	12
% reduction from base case		93	26	94	53		93	25	94	52
Case 5: Schools closed after 10 symptomatic cases, children and teenagers kept home, household contacts doubled, compliance 50%										
Average	100	1,551	135	90	47	95	1,630	141	94	49
SD		692	49	40	31		614	42	37	30
% reduction from base case		68	–67	87	–33		68	–72	87	–36
Case 6: Schools closed after 10 symptomatic cases, children kept home, household contacts doubled, compliance 90%										
Average	100	2,539	116	199	49	96	2,642	120	206	51
SD		661	30	66	17		433	23	56	14
% reduction from base case		48	–44	71	–38		47	–46	71	–40
Case 7: All with symptomatic cases stay at home, compliance 90%										
Average	100	3,692	91	408	41	94	3,926	95	433	43
SD		1,031	25	130	14		458	17	85	10
% reduction from base case		25	–12	41	–16		22	–16	38	–20

*Cases 2–7 are targeted social distancing strategies. Negative percent reductions reflect percent increases. Epidemics are defined as >100 infected. SD, standard deviation.

also more likely to become ill (*17*,*18*) as well as a person that is also more likely to be infected when exposed to children or teenagers than to adults (*14*).

Reasonable correspondence is observed (Figure 6) between age class–specific attack rates and those of past pandemics (*19*–*21*). Infections transmitted within each environment are also consistent with other simulation studies (*10*–*14*). The maximum value of the overall branching factor (Figure 5) reflects the often-cited reproductive number R_o. However, how R_o should be calculated from small-community data such as ours is ambiguous (*10*,*11*,*14*). To estimate R_o, we pooled results across 100 communities (simulations) to reflect a population of 1 million (Figure 5B). The maximum value of the bulk ratio (new infections to old) within the first 10 generations is 1.6, and we choose it as our estimate of R_o. An R_o of 1.6 with an attack rate of 50% matches recent pandemic simulation results (*10*,*14*) and lies within the range (1.5–1.7) for the 1957–58 influenza pandemic (Figure 5B) (*10*).

Base Case–Targeted Social Distancing

High infectiousness and a high number of contacts, many like-to-like, create a zone of high infectious contact centered on children and teenagers within the community's social network. Targeting this zone can protect the community at large.

First, we examined closing schools. Although contacts in classes are removed, those in all other groups may increase because children and teenagers now spend more time at home, in neighborhoods, with friends, and in public spaces. We assume that school closure at a minimum doubles household contacts. Closing schools with 90% compliance the day after 10 symptomatic cases reduces the attack rate by 22% (Table 2). However, if we assume that school closure doubles all link contact frequencies for children or teenagers within their nonschool groups, attack rates are increased by 18% (Table 2).

Alternatively, we send all children and teenagers home after school closure to remain for the duration of the pan-

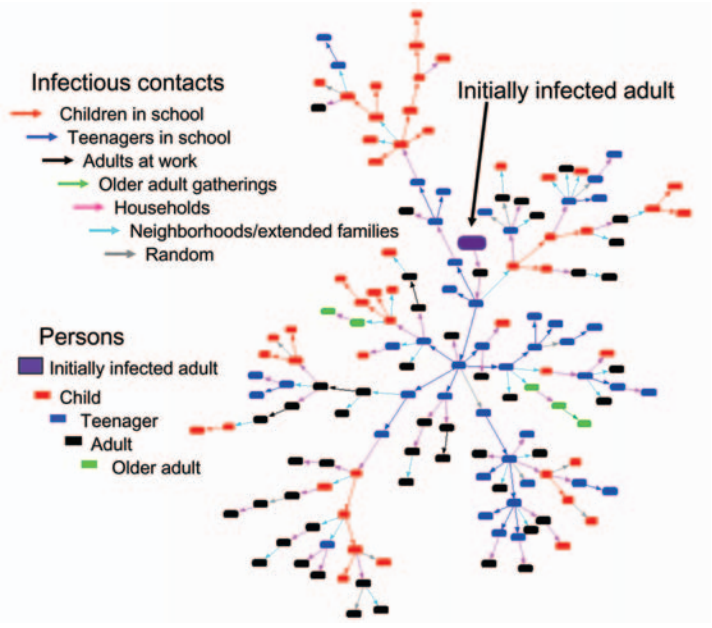

Figure 4. Initial growth of an infectious contact network. Colored rectangles denote persons of designated age class, and colored arrows denote groups within which the infectious transmission takes place. In this example, from the adult initial seed (large purple rectangle), 2 household contacts (light purple arrows) bring influenza to the middle or high school (blue arrows) where it spreads to other teenagers. Teenagers then spread influenza to children in households who spread it to other children in the elementary schools. Children and teenagers form the backbone of the infectious contact network and are critical to its spread; infectious transmissions occur mostly in the household, neighborhood, and schools.

demic. Now contact frequencies are reduced by 90% for all groups that contain only children or teenagers (classes and their random networks) and doubled, as before, for children or teenagers in households. In the extended family or neighborhood and the random overall networks, child or teenager contact frequencies are also reduced by 90%. Thus, although children and teenagers are restricted to the home, adults and older adults go about their day-to-day routines, except that they avoid children or teenagers who are not household members. Imposing this strategy the day after 10 symptomatic cases reduces attack rates by 93% (Table 2). Waiting until 80 symptomatic cases reduces attack rates by 73% (Figure 7A).

To evaluate the tradeoff between effectiveness and public compliance, we reduced the percentage of nonschool and nonhousehold contacts that have their frequencies reduced with the child and teenager stay-at-home policy (Figure 7B). At 50% compliance, attack rates can be reduced by 68% (Table 2). Reduction in compliance also increases the time scales for the epidemic. Epidemics

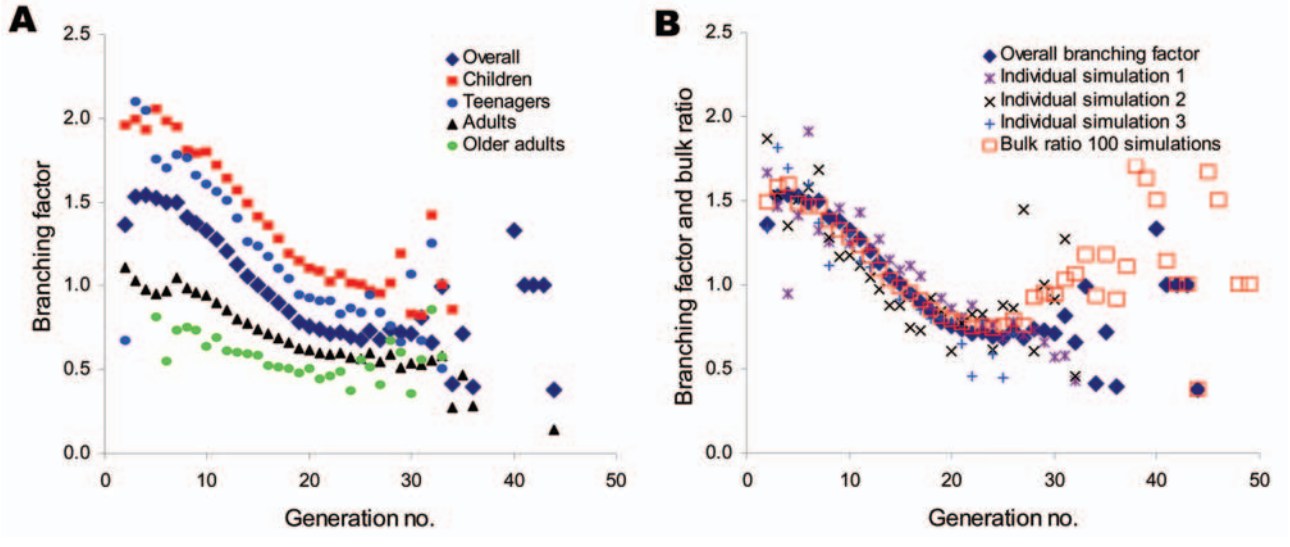

Figure 5. Branching factor and the approximation of the reproductive number R_o. A) Overall and age class–specific branching factors as a function of generation averaged over 100 simulations. The standard deviations of these averages can be large (≤0.72 at the peak value for teenagers) and reflect the heterogeneity within the person contact networks and from community to community. B) Branching factors for overall average and 3 example simulations compared with the bulk ratio of infections in a generation to those in the previous generation pooled across 100 simulations. We chose the maximum value of the bulk ratio (1.6) as an approximation of the reproductive number R_o.

Table 3. Unmitigated base case infectious contact fractions (% of the total no. of infectious contacts) between age classes*

Class	To children	SD	To teenagers	SD	To adults	SD	To older adults	SD	Total	SD
From children	18.6	0.8	2.9	0.3	16.1	1.1	1.2	0.2	38.8	2.4
From teenagers	2.4	0.8	9.1	0.7	8.0	0.5	0.6	0.1	20.1	2.1
From adults	6.0	0.6	3.8	1.2	26.0	5.9	2.1	0.4	38.0	7.9
From older adults	0.2	0.1	0.2	0.1	0.9	0.9	1.8	0.3	3.1	0.6
Total	27.3	2.2	16.0	2.2	50.9	7.7	5.8	0.9		

*SD, standard deviation.

lengthen above the base case and reach a factor of ≈1.8 at 40% compliance (Figure 7B).

Other social distancing strategies can be considered. Because children outnumber teenagers and children are more infective and susceptible, what happens if only children are distanced, while teenagers attend school and follow their usual routines? At 90% compliance, this strategy reduces attack rates by 47% (Table 2). What if all sick persons remain at home when symptomatic? At 90% compliance this strategy reduces attack rate by 20% (<25% of infectious persons are influenced as $pS \times pH = 0.25$ for adults only) (Table 2).

More Infective Strains and Contact Networks with Less Emphasis on the Young

We have modeled an influenza strain with an infectivity representative of the 1957–58 Asian influenza pandemic and a social contact network reflective of a stylized US

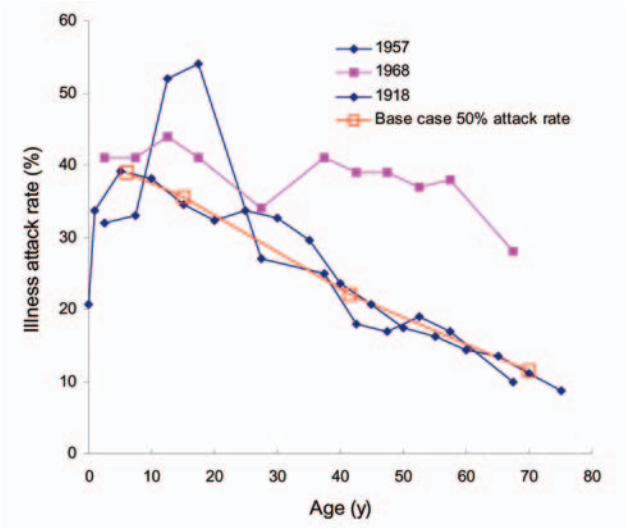

Figure 6. Comparison of simulated age class–specific illness attack rates with past pandemics. Simulated illness attack rates (half the infectious attack rate) for the unmitigated base case are close to those found in studies of historic pandemics in 1957 (*19*), 1968 (*20*), and 1918 (*21*). Notable differences are the 1968 Hong Kong flu, which had more emphasis on adults, and the 1957–58 Asian flu, which had more emphasis on youth; however, historic data are inherently uncertain. Closer correspondence to either of these 2 cases could be achieved through changes in I_A or S_A or modification of the underlying social contact network as it was likely different from that of a small town of today.

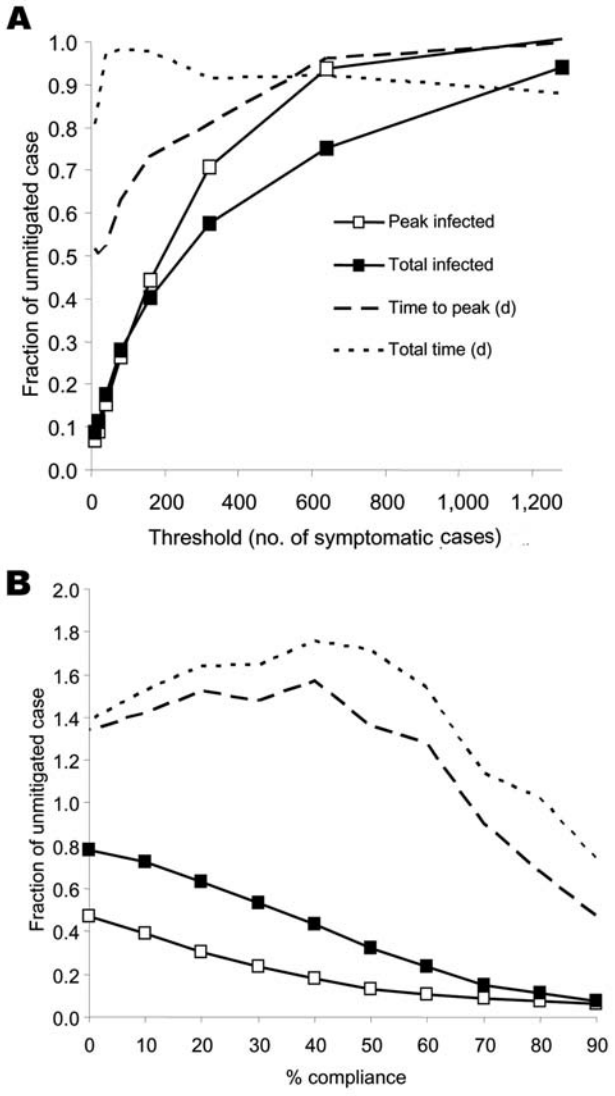

Figure 7: Fraction of unmitigated base case attack rate for targeted social distancing of children and teenagers as a function of A) implementation policy threshold given by the number of symptomatic cases (compliance at 90%) and B) compliance with staying at home (implementation policy threshold at 10 symptomatic cases, 0% compliance closes schools alone). Each point represents the average of simulations of 100 that yielded epidemics (>100 infected). Standard deviations for variation of threshold are <3% of the total population. However, for compliance variation, standard deviations increase to a maximum of 7% of the total population at a compliance of 30%.

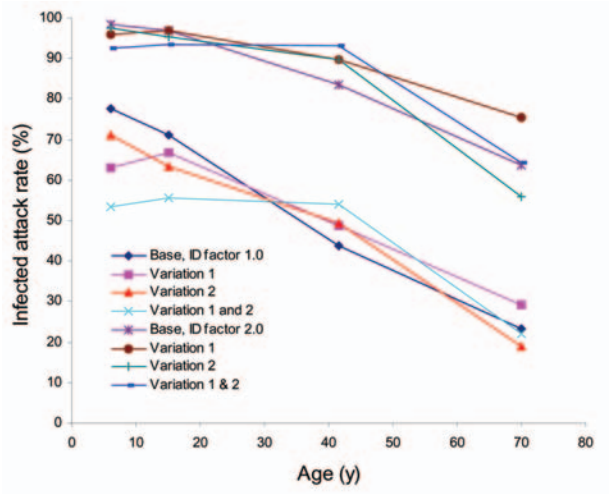

Figure 8. Unmitigated age-specific attack rate results for disease infectivity (I_D) factors of 1.0 and 2.0 and base case, variation 1 (removal of relative infectivity and susceptibility), variation 2 (increase in work group frequency of contact to give all children, teenagers, and adults the same overall contact frequencies), and variations 1 and 2 combined. Illness attack rates shown in Figure 6 are half these values.

town. Although results for the unmitigated base case yield age class–specific attack rates similar to those for past epidemics (Figure 6), will the targeted social distancing strategies found above remain effective if 1) the strain is more infective or 2) the importance of the young is deemphasized?

To explore these questions, we considered 3 increases in disease infectivity I_D by factors of 1.25 (attack rate ≈66%, $R_o \approx 1.8$), 1.5 (attack rate ≈75%, $R_o \approx 2.0$), and 2.0 (attack rate ≈86%, $R_o \approx 2.4$). These increases encompass and exceed the 1918–19 Spanish influenza pandemic (R_o 1.8–2.0) (*10*). We also sequentially removed enhanced transmission by children and teenagers and thus the zone of high infectious contact that we have designed social distancing strategies to target. We created 3 variations: the first removed relative infectivity and susceptibility enhancement of children and teenagers (I_A and S_A 1.0) (variation 1); the second increased frequency of contact within the work environment by a factor of 4 to give adults the same number of contacts as younger persons (variation 2); and the third combined variations 1 and 2. For each of the resulting set of 4 cases (base, variation 1, variation 2, and variation 1 and 2), I_D was altered slightly to maintain the reference of ≈50% infected attack rate for $R_o \approx 1.6$.

As I_D increases, age specific–attack rates increase (Table 4). As we remove differences in the number of contacts and/or the relative infectivity and susceptibility (I_A, S_A) between young and adults, the infected attack rates systematically shift from young persons to adults (Figure 8). These results suggest that for such situations, social distancing strategies must be devised that focus on more than children and teenagers alone.

To find effective targeted social distancing strategy combinations across the range of disease infectivity and infectious contact networks, we formulated 5 strategies and applied them individually and in combination:

Table 4. Unmitigated case results for R_o and average attack rates (%) for increasing I_D and base case, variation 1, variation 2, and variations 1 and 2 combined*

			Attack rates									
Type	I_D factor	R_o	Overall	SD	Children	SD	Teenagers	SD	Adults	SD	Older adults	SD
Base case	1.0	1.6	51	1.3	79	1.8	72	2.2	45	1.4	23	1.7
	1.25	1.8	66	1.1	90	1.0	85	1.4	61	1.4	36	2.0
	1.5	2.0	75	0.8	95	0.6	92	1.1	71	0.9	47	2.1
	2.0	2.4	86	0.5	99	0.4	97	0.6	84	0.7	64	1.7
Variation 1	1.0	1.5	52	1.7	65	2.1	68	2.3	50	1.9	30	2.1
	1.25	1.7	70	1.1	82	1.5	84	1.5	68	1.2	47	2.4
	1.5	1.9	80	0.8	90	1.0	91	1.1	79	0.9	60	2.1
	2.0	2.4	90	0.5	96	0.5	97	0.5	90	0.6	76	1.6
Variation 2	1.0	1.5	52	1.6	72	2.3	64	2.4	50	1.8	19	1.7
	1.25	1.8	68	1.1	87	1.3	81	1.6	68	1.3	31	1.7
	1.5	1.9	78	0.8	93	0.7	89	1.4	79	1.0	41	2.1
	2.0	2.3	88	0.5	98	0.4	96	0.7	90	0.6	57	2.0
Variations 1 and 2 combined	1.0	1.5	52	2.0	55	2.3	57	2.7	56	2.3	23	1.7
	1.25	1.8	70	1.1	74	1.8	76	1.9	75	1.2	37	2.0
	1.5	2.0	80	0.8	84	1.2	85	1.2	85	0.8	48	2.0
	2.0	2.4	90	0.4	93	0.6	94	0.9	94	0.5	65	1.8

*Variation 1 is removal of relative infectivity and susceptibility; variation 2 is an increase in work group frequency of contact to give all children, teenagers, and adults the same overall contact frequencies. Average attack rates accumulate over only those simulations that resulted in epidemics (>100 infected). R_O, reproductive number; I_D, disease infectivity; SD, standard deviation.

Table 5. Mitigated case average attack rates (%) for increasing I_D and base case, variation 1, variation 2, and variations 1 and 2 combined*

	Strategy combination					Base case I_D factor				Variation 2 I_D factor				Variation 1 I_D factor				Variations 1and 2 I_D factor			
No.	S	CTsd	AOAsd	LL	Wsd	1	1.25	1.5	2	1	1.25	1.5	2	1	1.25	1.5	2	1	1.25	1.5	2
1						51	66	75	86	52	68	78	88	52	70	80	90	52	70	80	90
2					Wsd	48	63	72	84	41	60	71	83	47	66	77	88	35	58	72	86
3				LL		41	57	67	79	37	57	68	82	36	57	70	84	28	55	69	84
4				LL	Wsd	39	55	65	78	30	49	62	77	30	53	67	82	12	42	60	78
5			AOAsd			38	51	59	70	40	58	68	79	25	46	58	72	33	56	69	80
6			AOAsd		Wsd	35	48	56	66	30	47	58	71	18	39	51	66	9	37	53	71
7			AOAsd	LL		32	46	55	66	28	48	60	73	13	36	50	67	11	40	57	74
8			AOAsd	LL	Wsd	30	43	52	63	21	40	51	66	10	32	46	62	4	23	42	64
9		CTsd				41	58	69	82	45	64	75	86	41	63	76	88	46	67	78	88
10		CTsd			Wsd	37	55	66	79	31	53	66	80	32	57	71	85	21	52	67	83
11		CTsd		LL		29	48	60	75	26	50	64	78	20	47	63	80	19	49	65	81
12		CTsd		LL	Wsd	27	45	57	72	16	40	55	72	14	41	58	77	6	32	53	74
13		CTsd	AOAsd			29	46	56	68	34	55	66	78	15	40	54	70	27	54	67	79
14		CTsd	AOAsd		Wsd	26	42	52	64	20	41	54	69	9	31	45	63	5	30	50	69
15		CTsd	AOAsd	LL		22	39	51	64	18	42	56	72	7	29	45	64	7	35	55	73
16		CTsd	AOAsd	LL	Wsd	20	37	48	61	10	32	47	63	5	22	39	58	3	16	37	61
17	S					41	61	73	85	45	66	77	87	47	68	79	90	51	69	80	90
18	S				Wsd	36	57	70	83	30	54	68	83	38	62	75	88	29	56	71	85
19	S			LL		23	47	62	78	23	49	65	80	20	50	66	83	22	51	67	83
20	S			LL	Wsd	19	44	59	76	9	38	55	74	13	44	62	80	6	35	55	76
21	S		AOAsd			26	47	59	74	34	56	69	81	16	44	60	76	34	58	70	82
22	S		AOAsd		Wsd	20	41	55	70	14	41	57	73	8	35	52	71	7	36	55	74
23	S		AOAsd	LL		11	35	51	68	12	40	57	74	5	28	48	69	8	38	57	75
24	S		AOAsd	LL	Wsd	9	32	47	65	5	27	45	66	4	20	41	64	3	14	39	65
25	S	CTsd				4	26	50	73	15	47	64	80	12	46	64	82	34	58	71	84
26	S	CTsd			Wsd	3	15	40	68	3	21	46	71	5	32	55	78	6	36	56	77
27	S	CTsd		LL		2	7	29	60	3	21	45	70	3	17	43	70	5	33	54	75
28	S	CTsd		LL	Wsd	2	6	20	54	2	6	24	57	2	9	31	64	2	9	33	64
29	S	CTsd	AOAsd			2	4	13	44	4	24	48	70	2	4	15	49	8	37	56	73
30	S	CTsd	AOAsd		Wsd	2	3	7	30	2	5	16	49	2	3	6	28	2	5	20	54
31	S	CTsd	AOAsd	LL		2	3	9	34	2	7	27	58	2	3	7	36	3	11	35	63
32	S	CTsd	AOAsd	LL	Wsd	2	3	6	25	2	3	8	37	2	2	5	20	2	3	9	39

*Variation 2 is an increase in work group frequency of contact to give all children, teenagers, and adults the same overall contact frequencies; variation 1 is removal of relative infectivity and susceptibility. I_D, disease infectivity; S, school closure; CTsd, children and teenagers social distancing; AOAsd, adults and older adults social distancing; LL, liberal leave; Wsd, work social distancing. Shaded numbers denote strategy combinations that reduce the attack rate to <10% of the population (illness attack rate <5%). Average attack rates accumulate over only those simulations that resulted in epidemics (>100 infected). Average standard deviation across the entire set of simulations was 2.2% with a maximum of 7.6%.

1) school closure (S) where the contact frequency within schools was reduced 90% and children and teenagers household contacts were doubled; 2) children and teenagers social distancing (CTsd) where their contact frequencies in all nonhousehold and nonschool groups were reduced 90% and their household contacts doubled; 3) adult and older adult social distancing (AOAsd), where their contact frequencies in all nonhousehold and nonwork groups were reduced 90% and household contacts doubled; 4) liberal leave (LL), where all children and teenagers and 90% of adults withdraw to the home when symptomatic; and 5) work social distancing (Wsd) where the contact frequency within work groups was reduced 50%. For each combination, we implemented the strategy(ies) the day after 10 symptomatic cases and conducted 100 simulations.

As I_D increases, more strategies must be combined to keep the attack rate <10% (Table 5, shaded values). As children and teenagers become less prominent, targeting adults becomes important, even at an I_D factor of 1. For an I_D factor of 1.5 (as infective as the 1918–19 Spanish influenza pandemic) and across all variations, both the young and adults must be targeted and all strategies must be implemented to effectively mitigate the epidemic. However, for an I_D factor of 2.0, we can at best reduce the attack rate to 20–40% through full strategy combination, not ideal but still a significant benefit.

Discussion

Results for our stylized small town suggest that targeted social distancing strategies can be designed to effectively mitigate the local progression of pandemic influenza

without the use of vaccine or antiviral drugs. For an infectivity similar to that of the 1957–58 Asian influenza pandemic, targeting children and teenagers, by not only closing schools but also by keeping these age classes at home, was effective. However, given uncertainty in the infectivity of the influenza strain, underlying social contact network, or relative infectivity/susceptibility of the young versus adults, planning to implement strategies that also target adults and the work environment is prudent. To mitigate a strain with infectivity similar to that of the 1918–19 Spanish influenza pandemic, simulations suggest that all young and adults must be targeted regardless of the likely enhanced transmission by the young.

Implementation of social distancing strategies is challenging. They likely must be imposed for the duration of the local epidemic and possibly until a strain-specific vaccine is developed and distributed. If compliance with the strategy is high over this period, an epidemic within a community can be averted. However, if neighboring communities do not also use these interventions, infected neighbors will continue to introduce influenza and prolong the local epidemic, albeit at a depressed level more easily accommodated by healthcare systems.

Our design approach explicitly implements disease-host interaction within the social contact network where the disease spreads. Measuring contact networks within communities for the spread of infectious diseases requires focused research that combines sociology, public health, and epidemiology. Such networks will likely differ across cultures, between urban and rural communities, and with community size. With the aid of detailed demographic data, expert elicitation of social scientists and community members, behavioral surveys, and possibly experiments, a network could be constructed for any community of interest. Configurations that consider, for example, college campuses or military reservations may be of use given that the highest death rate of any group in the 1918–19 Spanish influenza pandemic was in young adults (22).

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Seroprevalence of Hepatitis E Virus Infection, Rural Southern People's Republic of China

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Genotype 4 hepatitis E virus (HEV) is the dominant cause of hepatitis E in the People's Republic of China; swine are the principal reservoir. Our study was conducted in 8 rural communities of southern China, where families keep pigs near their homes. Phylogenetic analysis showed that 23 of 24 concurrent virus isolates from this region are genotype 4 strains. Among the study populations, immunoglobulin G anti-HEV seroprevalence accumulated with age at ≈1% per year for persons ≤60 years of age. After age 30 years, seroprevalence increased at higher rates for male than for female study participants. The overall seroprevalence was 43% (range 25%–66%) among the communities. Infection rates were higher for participants between 25 and 29 years of age. The results suggest that HEV infection probably has been endemic in southern China for at least 60 years, with swine being the principal reservoir of human HEV infection in recent years.

Hepatitis E virus (HEV), a member of the *Hepevirus* genus, is an RNA-positive strand virus that resembles calicivirus both morphologically and in organization of its 7.5-kb genome (*1*). The virus was first identified as the cause of extended waterborne outbreaks of hepatitis, with significant deaths among pregnant women (*2*). Widely distributed in nature, the virus is detected in swine and other animals—both domestic and wild (*3*–*5*). Based on phylogenetic analysis, the virus isolates can be separated into 4 major groups, genotypes 1–4 (*6*). While viruses of genotypes 1 and 2 are isolated exclusively from humans, those from genotypes 3 and 4 have also been isolated from swine and other animals (*7*). Genotype 1 strains are mainly distributed in Asia and the Middle East (*8*,*9*), where they frequently cause waterborne outbreaks of hepatitis (*10*,*11*). Genotype 2 virus was first detected in Mexico (*12*). Genotype 3 strains are widely distributed; they have been isolated from swine in North America (*13*), South America (*14*), Europe (*15*), Oceania (*16*), and Asia (*17*) and, in some of these areas, from rare, indigenous human cases of hepatitis E (*18*,*19*). Genotype 3 virus isolated from wild pigs and deer in Japan was recently found to be the cause of an outbreak of foodborne infection (*20*,*21*). Genotype 4 is largely restricted to Asia. The virus was detected in archival swine serum samples in India collected in 1985 (*22*) and since then has been detected in swine in Taiwan (*23*), mainland People's Republic of China (*5*), Indonesia (*24*), India (*22*), and Japan (*25*). Genotype 4 virus causes sporadic cases and is associated with foodborne infection but has not been generally associated with waterborne outbreaks. The different HEV genotypes are indistinguishable serologically (*26*), however, and studies in primates show cross-protection among the 4 genotypes (*27*,*28*).

HEV was first detected in People's Republic of China during an extended outbreak in Xinjiang Province in 1986 (*10*). The causal agent was a strain of genotype 1 virus, which persisted as the dominant genotype in China until 2000, when genotype 4 emerged as the dominant genotype (*29*,*30*). A recent study conducted in 2 swine farming districts of eastern China showed that genotype 4 virus freely circulates among swine and humans: an estimated 9% of swine and 0.3% of humans have asymptomatic infection

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(*31*). In seroepidemiologic studies conducted concurrently by these authors, the risk for human HEV infection was associated with occupational contact with swine and swine sewage, making the animals a principal reservoir for human infection.

We describe a seroepidemiologic study of HEV infection in 8 rural communities of southern China and a phylogenetic analysis of the virus circulating in the region. The results showed that HEV infection is endemic in the region and probably has been for at least 60 years. The prevalent virus population is genetically diverse, although dominated by genetically diverse genotype 4 virus.

Materials and Methods

Study Participants

We conducted a cross-sectional and a follow-up seroepidemiologic study of HEV infection in 2003 and 2004 in conjunction with annual health examinations conducted among residents of rural communities in Guangxi Province (GX) in southern China. These exams involve routine clinical examination, routine biochemical testing, and determination of hepatitis A virus (HAV) and hepatitis B virus (HBV) serologic status. Participants were enrolled in the study after providing informed consent; parental consent was obtained for participants <16 years of age. A questionnaire was used to record demographic data, education level, employment, source of water supply, sanitation practices, and household contact with pigs and poultry. Ethical approval for the study was obtained from the Guangxi Institutional Review Board.

Immunoglobulin G (IgG) anti-HEV Assay

An aliquot of serum was obtained from the samples taken for routine biochemical testing and HAV and HBV status and made available for the present study. Serum samples were tested for IgG anti-HEV by using a commercial ELISA (Wan Tai Pharmaceutical Co., Beijing, China), produced with a recombinant peptide corresponding to amino-acid residues 396 to 606 of the major structural protein specified by open reading frame 2 (ORF2) of the HEV genome (*32*). Serum samples were diluted 1:10, and tested according to manufacturer's instruction. A positive reaction was indicated when the signal:cut-off (S:CO) exceeded 1.5.

Detection of HEV RNA

For the virologic study, serum samples were taken from 24 patients admitted to local hospitals in 2003 and 2004 with serologically diagnosed hepatitis E. Total RNA was extracted from 250 μL of sample with Trizol (Invitrogen). Reverse-transcription polymerase (RT)-PCR was performed as described previously (*33*,*34*). Briefly, a 150-nt segment of ORF2, was amplified with primers E1 (5′-CTGTTTAA[C/T]CTTGCTGA CAC-3′,6,260–6,279) and E5 (5′-[A/T]GA[A/G]AGCCAAAGCACATC-3′, nt 6,568–6,551) in the first round of PCR and primers E2 (5′-GACAGAATTGATTTCGTCG-3′, nt 6,298–6,316) and E4 (5′-TG[C/T]TG GTT[A/G]TC[A/G]TAATCCTG-3′, nt 6,486–6,467) in the second round. PCR cycling conditions for both rounds consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 40 s. PCR products were purified and sequenced in a forward and reverse direction by using an automatic nucleotide sequencer (ABI model 3730 sequencer, Applied Biosystems, Foster City, CA, USA).

Phylogenetic Analysis

A 150-nt ORF2 segment was amplified from 24 virus isolates from patients with serologically diagnosed acute hepatitis E who were admitted to local hospitals during the period of study. The nucleotide sequence of the amplified products and that of prototypes of different genotypes of HEV strains were aligned by using the MEGA 3.0 software (version 3.0, www.megasoftware.net). Genomic sequences of prototype HEV strains (Burma1, M73218; Burma2, D10330; CN-Xinjiang, D110920; CN-genotype4, AJ272108; India1, X98292; Pakistan, M80581; Mexico, M74506; U.S., AF060668) were obtained from GenBank. Phylogenetic trees were generated by the minimum evolution method and the interior branch method; 1,000 resamplings of the data were used to calculate percentage of the branches obtained. The identity between the nucleotide sequences was calculated by using the program MegAlign (DNAstar package version 5.03; Lasergene, DNAstar Inc., Madison, WI, USA).

Statistical Methods

Seroprevalence was standardized for age and sex according to China's national census of 2000. Multivariate unconditional logistic regression analysis was performed with SAS (Version 8.2; SAS Institute Inc., Cary, NC, USA) and was used to identify independent determinants of IgG anti-HEV prevalence and to estimate the level of the associated risk (*35*).

Results

Serum samples were taken in 2003 from 7,284 participants; a second sample was taken 12 months later from a subpopulation of 3,431 of these persons (Table 1). The study participants were recruited from 8 rural communities situated 50–500 km of one another in southern China. The water supply for this area comes from wells, rivers, and streams. Tap water and sewage treatment are not generally available. The study population was of similar socioeconomic and cultural backgrounds, and, in general, participants had

Table 1. Study participants*

Community	2003 n	2003 Age (y) Mean	2003 Age (y) SD	2003 Age (y) Range	2003 Sex ratio (M:F)†	2004 n	2004 Age (y) Mean	2004 Age (y) SD	2004 Age (y) Range	2004 Sex ratio (M:F)
Linyun	1,047	24.1	15.4	1–74	0.90	738	22.9	15.4	1–72	0.96
Xin'an	1,037	31.3	17.9	1–87	0.85	533	31.8	18.4	1–87	0.77
Guilin	542	35.3	15.0	0.9–80	1.14	340	37.3	14.1	1–80	1.06
Luochen	981	32.5	19.0	1–85	0.73	455	34.2	19.7	1–79	0.72
Tiendan	377	35.1	16.4	1–70	0.98	166	37.2	16.4	2–70	0.73
Binyan	1,106	28.9	18.2	1–78	0.92	657	28.5	19.1	1–78	0.84
Linshan	1,230	27.4	20.5	0.4–87	0.94	542	26.2	21.6	0.5–80	0.97
Liuzhen†	964	44.6	16.8	0.1–79	0.88	–	–	–	–	–
Total	7,284	31.7	18.8	0.1–87	0.89	3,431	29.5	18.8	0.5–87	0.87
M	3,440	30.2	19.4	0.4–85	–	1,597	27.9	19.3	0.5–80	–
F	3,844	33.0	18.8	0.1–87	–	1,834	30.9	18.3	1–87	–

*Serum samples were taken in 2003 from 7,284 persons residing in 8 communities of Guangxi Province. Second samples were taken 12 mo later from a subpopulation of 3,431 study participants residing in 7 of these communities. *SD, standard deviation;, M = male; F = female.
†Dash indicates "not done" in reference to Liuzhen and "not applicable" in reference to sex.

been residing in their respective communities for most of their lives. Farming is the major source of income; most families rear swine and other domestic animals for their own consumption and for sale to supplement family incomes. Within these communities, men commonly migrate to urban centers to seek employment; hence, female participants predominate in the study population.

Table 2 shows the IgG anti-HEV status of the 7,284 study participants from these 8 rural communities in 2003. The age and sex-standardized IgG anti-HEV seroprevalence is 43.5% for the general study population and 25.2%–66.1% for the 8 communities. The age-standardized seroprevalence is 47.1% for male participants and 39.7% for female ones. Multivariate unconditional logistic regression analysis identified age, sex, and community to be independent determinants for IgG anti-HEV seroprevalence, but not other factors such as income, employment, education, and source of water supply. Because rearing pigs and chickens adjacent to one's home is common, most families reside in close proximity to domestic animals whether or not they keep them themselves. Consequently, assessing the risk for infection attributable to this practice was not possible.

On the basis of results obtained with the samples taken in 2003, the age-specific IgG anti-HEV seropositive rate was calculated in 5-year increments up to 69 years of age and summarily for older participants. The IgG anti-HEV seropositive rate of the general population (Figure 1A) accumulated with age for both male and female participants (Figure 1B). The seropositive rate of the general study population increased at a relatively constant rate of ≈1% per year up to ≈60 years of age, and then remained essentially stable. IgG anti-HEV seropositive rate increased at a similar rate for male and female participants <30 years of age then at higher rate for male participants 30–59 years of age (Figure 1B). Multivariate unconditional logistic regression analysis estimated that IgG anti-seropositive rates were similar for male and female participants up to age 30 years (odds ration [OR] = 0.9, 95% confidence intervals [CI] = 0.7–1.1) but were ≈2-fold higher for male participants >30 years of age (OR = 2.1, 95% CI = 1.7–2.6).

Changes in IgG anti-HEV serologic status occurring in 2004 were determined by using a second sample taken 12 months later from a subpopulation of 3,431 participants (Table 3) from 7 of the 8 communities. The results showed that 4.3% of seronegative participants seroconverted to positive, and 1.4% of seropositive participants underwent negative seroconversion. The overall seropositive rate of the subpopulation increased from 46.2% in 2003 to 49.1% in 2004. The positive seroconversion rate was higher for male participants, but the difference was not statistically significant ($p>0.05$). One community (Linyun) evidently experienced an undetected outbreak of HEV infection between 2003 and 2004, since its positive seroconversion rate of 17.9% was significantly higher than the positive

Table 2. IgG anti–hepatitis E virus (HEV) seroprevalence in rural communities, southern China, 2003

Communities	Participants	IgG anti-HEV seroprevalence (%)* Observed	Standardized
Linyun	1,047	58.4	66.1
Xin'an	1,037	60.4	59.4
Guilin	542	57.8	49.4
Luochen	981	42.2	42.6
Tiendan	377	45.6	38.8
Binyan	1,106	31.1	36.2
Linshan	1,230	21.0	25.2
Liuzhen	964	43.1	30.4
Total	7,284	43.3	43.5
Male	3,440	45.8	47.1
Female	3,844	41.0	39.7

*Seroprevalence standardized according to People's Republic of China national census of 2000; IgG, immunoglobulin G.

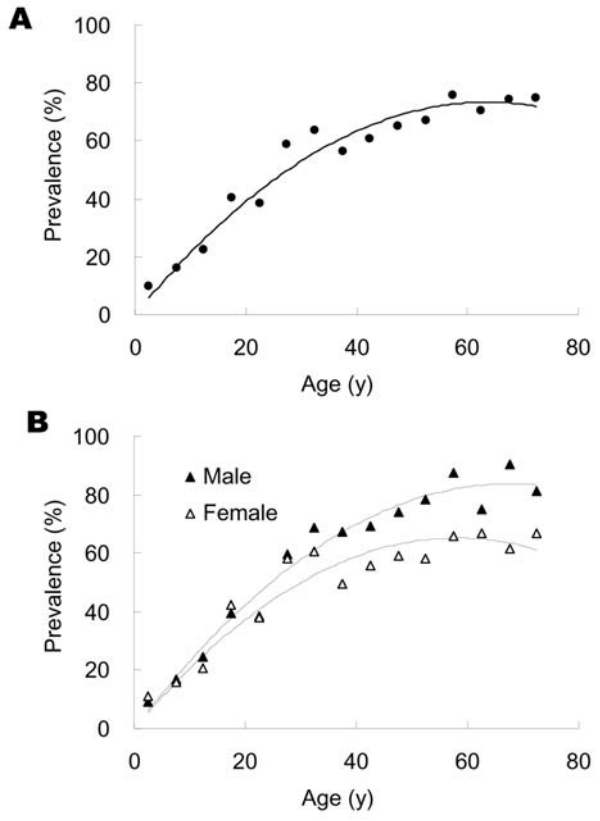

Figure 1. Age specific anti–hepatitis E virus (HEV) seropositive rates in a study population. Age-specific immunoglobulin G anti-HEV seropositive rates for (A) both sexes or (B) either sex separately (▲ for male study participant, △ for female) were determined for every 5 years from 0 to 69 years of age and for older participants, using samples taken in 2003 from 7,284 persons.

seroconversion rates of the other communities ($p<0.01$). In those communities, positive seroconversion rates ranged from 0.7% to 4.2%, but the differences between them were not statistically significant ($p>0.05$). The negative seroconversion rates were 0%–3.03% among the different communities, also not statistically significant ($p > 0.05$).

Age-specific IgG anti-HEV positive seroconversion rates and negative seroconversion rates were calculated in increments of 5 years up to 59 years of age (for 2003, it was 69 years) and then summarized for the older participants. Figure 2A shows that positive seroconversion occurred at all ages. Highest rates occurred among persons in the 25- to 29-year age group (11.8% per year) and ranged from 2.1% to 5.6% per year for the other age groups. On the basis of multivariate unconditional logistic regression analysis, the risk for infection was ≈3-fold higher for persons 25–29 years of age than for those in the other age groups (OR = 3.2, 95% CI = 1.7–5.8).

The overall rate of negative seroconversion was 1.4%. For the 0–4 year age group, this rate was 18.8%, which is ≈15 times higher that of the older age groups (OR = 14.9, 95% CI = 4.9–45.4); however, this rate likely reflects the loss of maternal antibodies. Among the older age groups, negative seroconversion occurred at similar rates of 0 to 3.6% per year.

All HEV infections observed among the study participants were asymptomatic; no cases of overt hepatitis E were observed. To study HEV prevalence in the communities during our study period, we obtained 24 HEV strains isolated from persons with serologically diagnosed hepatitis E who were admitted to hospitals in Guangxi between 2003 and 2004. Figure 3 shows the phylogenetic tree produced with the alignments of a 150-nt ORF2 sequence. All 24 isolates had distinct nucleotide sequences. One isolate was a genotype 1 virus, which is more closely related to prototype Chinese than to Burmese, Indian, or Pakistani genotype 1 strains. The other 23 isolates were genotype 4 virus related to the Japanese prototype genotype 4 strain. Genotype 4 isolates are genetically diverse, and in our study genetic identity between pairs of isolates ranged from 88.7% to 99.3%, with a mean value of 95.4%.

Table 3. Changes in IgG anti-hepatitis E virus (HEV) status in rural communities of southern China, 2003–2004

Communities	Participants	IgG anti-HEV seroprevalence (%)		PC*	NC*
		2003	2004	% (n)	% (n)
Linyun	738	62.2	78.2	17.9 (50)*	2.0 (9)
Xin'an	533	63.6	65	3.1 (6)	0.3 (1)
Guilin	340	58.2	59.4	4.2 (6)	3.0 (6)
Luochen	455	44.4	44.9	2.0 (5)	1.5 (3)
Tiendan	166	48.2	46.9	1.2 (1)	2.5 (2)
Binyan	657	30.6	31.8	1.8 (8)	0.5 (1)
Linshan	542	19.4	20.1	0.7 (3)	0.0 (0)
Liuzhen†	–	–	–	–	–
Total	3,431	46.2	49.1	4.3 (79)	1.4 (22)
Male	1,597	48.5	52.1	5.0 (41)	1.4 (11)
Female	1,834	44.1	46.5	3.7 (38)	1.4 (11)

*Changes in serologic status were indicated by changes in IgG anti-HEV seroprevalence observed in 2003 and 2004, percent and number (n) of seronegative participants undergoing positive seroconversion (PC), and that of seropositive persons undergoing negative seroconversion (NC). Linyun has significantly higher PC ($p<0.01$).
†–, not done.

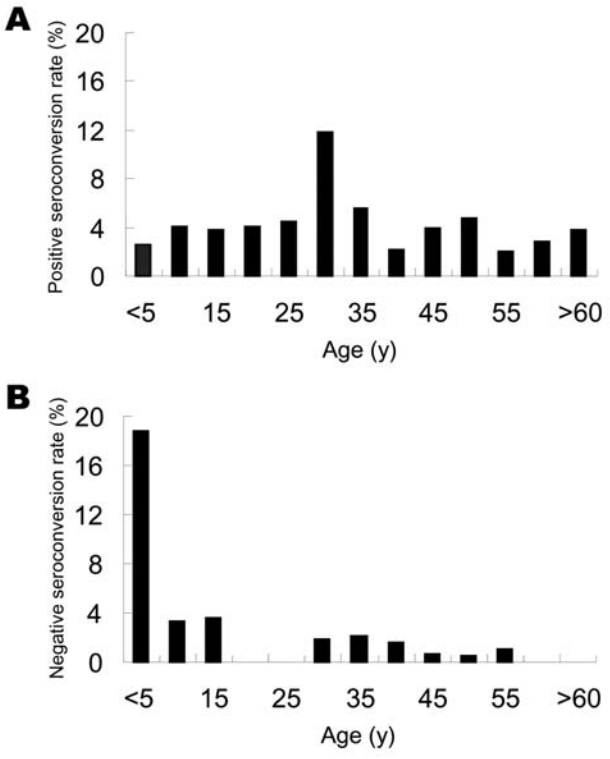

Figure 2. Changes in immunoglobulin G (IgG) anti-hepatitis E virus (HEV) serologic status, 2003–2004. Age-specific IgG anti-HEV–positive seroconversion (A) and age-specific IgG anti-HEV–negative seroconversion (B), determined for every 5 years of age from 0 to 59 years of age and in older participants, using samples taken 12 months apart from a subpopulation of 3,431 persons.

Discussion

We undertook a cross-sectional seroepidemiologic study in 2003 to determine the extent of HEV infection occurring in 8 rural communities in southern China. To determine new infections, we obtained a second serum sample from a subgroup of the 2003 study population after 12 months. Genetic analysis was performed on 24 strains of HEV isolated from patients with serologically diagnosed hepatitis E who were admitted to hospitals in the region during the study period.

The IgG anti-HEV assay used in the study was produced with a recombinant peptide of the major HEV structural protein that occurs naturally as a homodimer (*36*). The 3-dimensional structure of the homodimer appears to model the HEV neutralization sites and other important HEV antigenic determinants located on the virus capsid (*34*,*37*). Consequently, IgG anti-HEV determined by this assay correlated with protective immunity (*27*,*38*). The antibody is regularly detected in acute- and convalescent-phase serum samples from hepatitis E patients and persists in primates for more than a year after HEV challenge (*32*). In our study, the average negative seroconversion rate of the antibody produced in response to asymptomatic HEV infection was 1.4% per year. This shows that the IgG antibody determined by this assay is stable and could provide a reliable epidemiologic marker to study HEV infection.

Phylogenetic analyses were conducted by using alignments of a 150-nt ORF2 sequence of 24 isolates from serologically confirmed hepatitis E cases. Based on previous studies (*6*,*33*), phylogenetic relationships established on the basis of partial sequences, such as this, are expected to be similar to relationships established from a complete sequence of the viral genome. The 24 virus strains analyzed from hepatitis E patients admitted to local hospitals during the time of the study are generally representative of the virus population prevalent in southern China where our study took place. All 24 isolates are genetically distinct and consist of 1 genotype 1 isolate and 23 genotype 4 isolates.

Zheng et al (*31*) studying HEV infection in 2 swine farming districts of eastern China showed that most HEV infections were genotype 4, which freely cocirculates among swine and humans. Since viral burden is much higher in swine than in humans, the authors concluded that swine were the principal source of genotype 4 virus for human infection. This contention is supported by seroepidemiologic findings, which suggested that infection may be acquired by contact with these animals or their sewage. Swine may be postulated to represent the principal

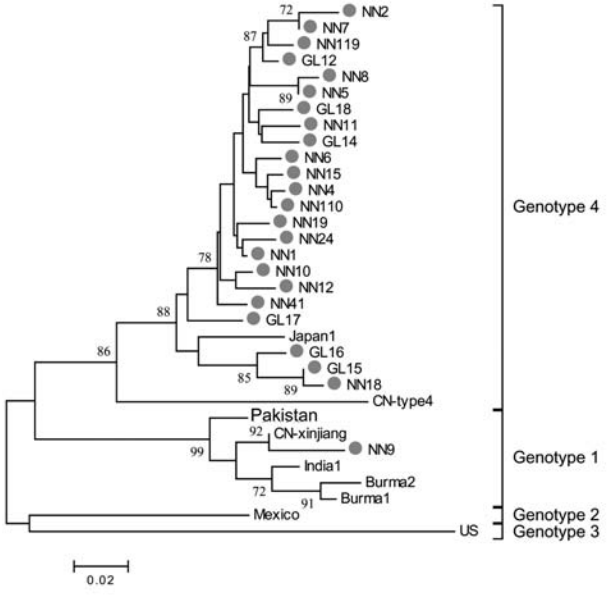

Figure 3. Phylogenetic analysis of hepatitis E virus (HEV) isolates. HEV isolates from patients with serologically diagnosed hepatitis E cases admitted in 2003 and 2004 to local hospitals are represented by closed circles. Prototype strains of indicated genotypes are designated according to site of isolation. Numbers on the branches represent (percent) reproduced values calculated from 1,000 resamplings of the data. The bar represents a genetic distance of 0.02-nt substitution per position.

reservoir of HEV in this current study area also because the prevalent virus population almost entirely comprises genotype 4 isolates and virtually all families raise pigs near their homes. According to this view, the level of infection among humans in each community would be determined primarily by the levels of infection of its swine population. Since the animals are kept close to family homes, humans could be the principal means by which the infection is spread among communities. However, the levels of infection differ substantially among communities, ranging from 25% to 66%, and a silent outbreak occurring in 1 of the communities during the time of the study involving 18% of its population did not spread to neighboring communities. This finding suggests that compared with swine, humans may not be an efficient vector for spreading genotype 4 virus infection.

The cross-sectional seroepidemiologic study showed that HEV infection is endemic in southern China. The average IgG anti-HEV seroprevalence of the population is 43%; participants' ages, sex, and communities are independent determinants. Seroprevalence was found to increase with age at a relatively constant rate of ≈1% per year until 59 years of age, and then remained essentially constant for older age groups. This finding suggests that HEV infection might have been endemic in southern China for > 60 years, so that persons in the 55- to 59-year age group may have been subjected to the same cumulative life-long exposure as older age groups. This estimate is consistent with findings from archival serum samples which indicate that HEV was prevalent in India before 1955 (*39*).

Our study identified 2 additional risk factors relating to the life style of the study participants. Seroprevalence was ≈2-fold higher for male participants than for female ones after 30 years of age but was similar for both sexes in the younger age groups. This finding probably reflects different roles adopted by males and females once families are established. Moreover, based on results from the follow-up study, the positive seroconversion rate is ≈3-fold higher among those 25–29 years of age than at other ages, which may be related to increased socialization during this stage of life.

In summary, our study showed that HEV infection is endemic in southern China and may have been so for at least 60 years. The prevalent virus is dominated by genetically diverse genotype 4 viruses. Among our study participants, infections seem to be mainly acquired from contact with swine; human-to-human transmission was of secondary importance. Herd immunity is built up separately to differing levels among different communities, leaving substantial proportions of the population vulnerable to HEV. Under such settings, vaccination programs for humans and swine could serve to boost herd immunity among humans and reduce the viral burden of swine herds.

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Spatiotemporal Analysis of Invasive Meningococcal Disease, Germany

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Meningococci can cause clusters of disease. Specimens from 1,616 patients in Germany obtained over 42 months were typed by serogrouping and sequence typing of PorA and FetA and yielded a highly diverse dataset (Simpson's index 0.963). A retrospective spatiotemporal scan statistic (SaTScan) was applied in an automated fashion to identify clusters for each finetype defined by serogroup variable region (VR) VR1 and VR2 of the PorA and VR of the FetA. A total of 26 significant clusters ($p \leq 0.05$) were detected. On average, a cluster consisted of 2.6 patients. The median population in the geographic area of a cluster was 475,011, the median cluster duration was 4.0 days, and the proportion of cases in spatiotemporal clusters was 4.2%. The study exemplifies how the combination of molecular finetyping and spatiotemporal analysis can be used to assess an infectious disease in a large European country.

Infection with meningococci in a susceptible human host can involve septicemia and meningitis, which are referred to as invasive meningococcal disease (IMD). IMD generates public concern and panic because of its often lethal outcome, its propensity to affect the young, and its occasional appearance in clusters. Meningococci are highly variable bacterial pathogens, as shown by a multitude of different sequence types identified by multilocus sequence typing (MLST) (*1*) and by antigen sequence typing of the outer membrane proteins such as PorA (*2*) and FetA (*3*).

Use of DNA sequence-based typing has several advantages over serotyping: information is reproducible and portable, most isolates are typeable, and culture-independent typing is possible. The consistent use of DNA sequence typing at the German National Reference Center for Meningococci (NRZM) since December 2001 has resulted in an extensive database containing a large number of unambiguously typed isolates. We define the term finetype as the antigenic profile of a meningococcal strain consisting of the serogroup, the sequence types of the variable regions (VRs) VR1 and VR2 of the PorA, and the sequence type of the immunodominant VR of FetA. The European Monitoring Group on Meningococci recommended in 2005 that PorA sequence typing be implemented as a standard typing method in all participating countries by 2007.

A meningococcal disease cluster is regarded as an aggregation of cases caused by the same bacterial strain closely grouped in space and time. While most cases of IMD appear in a sporadic fashion in industrialized countries, coprimary (i.e., occurring within 24 hours) and secondary cases occur regularly (*4*), as shown in institutional and household surveys (*5*). A community outbreak (*6*) represents an excess of incidence in a defined geographic area or population, in which direct links between cases are not always readily apparent. In most instances, detection of increases in case counts within defined spatial and temporal boundaries, for lack of more objective methods, must rely on the attentiveness of public health officials (*7*). Computer-assisted spatiotemporal cluster analyses might help identify and statistically evaluate increased instances of meningococcal disease, thus providing valuable information for further public health investigation and intervention.

Many methods have been developed for cluster analysis (*8*). A stochastic model has been applied to predict outbreaks of meningococcal disease in closed communities such as military cohorts (*9*). Hoebe et al. used space-time nearest neighbor analysis to statistically evaluate clusters of IMD in the Netherlands (*10*). One of the most widely used software packages for cluster analysis is SaTScan, which was developed by Martin Kulldorff (National Cancer Institute, Bethesda, MD, USA) and Farzad

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Mostashari (New York Department of Health and Mental Hygiene, New York, NY, USA) (*11*). In infectious disease epidemiology, SaTScan has been used to study listeriosis (*12*), methicillin-resistant *Staphylococcus aureus* infection (*13*), gonorrhea (*14*), West Nile fever (*15*), Creutzfeldt-Jakob disease (*16*), bovine respiratory syncytial virus (*17*), and pediatric pneumonia (*18*). Furthermore, national bioterrorism syndromic surveillance (*19*) and public health systems (*20*,*21*) rely on the use of this program. We applied SaTScan to a rigorously typed strain collection to identify and quantify finetype-specific clusters of cases of IMD in a large central European country with endemic meningococcal disease.

Materials and Methods

Data Collection

Meningococcal strains and culture-negative specimens obtained from patients with IMD are referred to NRZM by regional laboratories and hospitals for finetyping and, where applicable, antimicrobial drug resistance testing. Data are managed at NRZM by using a Microsoft Access database (Microsoft Corp., Redmond, WA, USA) that provides a user-friendly entry and retrieval surface. From December 1, 2001, to June 1, 2005, meningococci were detected from a normally sterile site in 1,828 patients; 1,616 patients with complete typing data and available residential postcode were included in the analysis. In 46 (2.8%) of these 1,616 cases, only clinical material (i.e., cerebrospinal fluid or serum) was analyzed. Of the remaining patients, no postcodes were available, no meaningful geographic information could be extracted from the postcode provided by the submitting laboratory, or finetyping could not be performed. Although the first 2 causes were outside the realm of NRZM, the last cause was mainly due to limitations of DNA sequence typing of meningococcal DNA from native samples such as cerebrospinal fluid or blood. A recent capture-recapture analysis for 2003 showed that NRZM processes samples from ≈65% of all cases estimated to occur in Germany (*22*). Underreporting to the NRZM occurs because submission of data by regional laboratories is voluntary, whereas reporting to the Robert Koch-Institute is statutory. For assessment of the systematic bias introduced by reporting behavior, see Adjustment for Potential Confounders in the Results section.

During the time of the study, no general recommendation existed in Germany for serogroup C conjugate vaccination. Two limited vaccination campaigns were initiated after observation of clusters 11 and 19 reported herein (Table).

Meningococcal Typing

Serogrouping of meningococcal isolates was accomplished by slide agglutination with monoclonal antibodies NmA 932, NmB 735, NmW135 1509, and NmY 1938 (Chiron-Behring, Marburg, Germany) and *Neisseria meningitidis* group C agglutinating sera (Remel, Lenexa, KS, USA). Culture-independent genotyping of meningococci was performed by amplification of polysialyltransferase genes specific to the serogroups B, C, W-135, and Y (*23*). Finetyping was accomplished by amplification and DNA sequencing of VR1 and VR2 of the *porA* gene encoding PorA and the VR of the *fetA* gene encoding the FetA protein (*2*,*3*). A finetype is expressed by the antigenic profile serogroup: P1.VR1,VR2:FVR1, where P1 is PorA and F is FetA. Deduced amino acid sequences were compared with entries in databases accessible at www.neisseria.org, which is curated by Keith Jolley (Oxford, UK) and Ian Feavers (Potters Bar, UK). Sequence data were analyzed with LASERGENE sequence-analysis software (DNASTAR, Madison, WI, USA) and TraceEdit Pro (Ridom, Würzburg, Germany).

DNA from culture-negative cerebrospinal fluid, blood, or serum was extracted by using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). Sensitive PCR protocols have been developed and validated to amplify serogroup-specific polysialyltransferase genes, and the variable regions of *porA* and *fetA* from culture negative specimen (data not shown). The discriminatory power of the typing methods was assessed by using the numeric index of the discriminatory ability derived from the Simpson index of diversity (*24*). The 95% confidence intervals (CIs) for the numeric indices were calculated as described by Grundmann et al. (*25*).

SaTScan Spatiotemporal Analysis

Information about SaTScan version 5.1.1 software is available at http://www.satscan.org. The program applies a likelihood function to circular windows originating at defined locations of increasing size and compares observed and expected case numbers inside and outside the scan window to detect clusters that are least likely to have occurred by chance. The statistical significance for each cluster is obtained through Monte Carlo hypothesis testing, i.e., results of the likelihood function are compared for a large number of random replications of the dataset generated under the null hypothesis. In this study, cases were assumed to be Poisson distributed in each location and the program's space-time scan statistic was applied. A user-friendly interface was programmed in Visual Basic for Applications, operating from within the database of NRZM: it handled the automatic data transfer to SaTScan and the creation of concise reports after completion of the analysis. Duplicate samples were identified and excluded

Table. Clusters of invasive meningococcal disease detected by SaTScan analysis, Germany, December 2001–June 2005

Cluster	Finetype	Cases	States (counties)*	Population	Year	Duration (d)	p value†	p_{age} value‡
1	Y:P1.5–2,10–28:F4–1	2	BY (1)	213,603	2002	21	0.003	0.003
2	B:P1.7–2,4:F3–3	2	NI (2)	2,286,265	2002	4	0.002	0.003
3	B:P1.18–1,30:F3–3	2	HH (1), NI (1)	3,096,084	2002	23	0.023	0.028
4	B:P1.5–1,2–2:F1–5	2	NI (1)	206,304	2002	18	0.006	0.004
5	B:P1:18,25–1:F5–1	2	TH (1)	142,595	2003	16	0.011	0.01
6	B:P1.5–2,10:F5–1	3	HE (1), RP (1)	2,394,079	2003	17	0.026	0.023
7	C:P1.5,2:F1–7	2	SL (1)	349,102	2003	3	0.035	0.033
8	B:P1.7,16:F5-X§	2	BY (2)	913,368	2003	10	0.028	0.025
9	C:P1.22,9:F3–3	3	NW (2), RP (1)	5,441,714	2003	2	0.002	0.002
10	C:P1.5,2:F3–3	4	BB (1), SN (1)	339,185	2003	18	0.004	¶
11	C:P1.5–1,10–8:F3–6	2	NW (1)	429,832	2003	4	0.008	0.011
12	C:P1.5–1,10–8:F4–1	2	BW (1)	134,407	2003	13	0.028	0.043
13	C:P1.5,2:F1–1	2	NW (2)	860,407	2003	1	0.037	0.032
14	B:P1.5–1,2–2:F1–14	2	MV (1)	120,959	2003	<1	0.001	0.001
15	C:P1.5,2:F5–8	3	NW (1), RP (2)	2,768,981	2003	1	0.001	0.005
16	W135:P1.5,2:F1–1	2	BW (2)	2,761,536	2003	2	0.044	*0.056*
17	B:P1.7,16:F3–3	2	MV (1)	52,994	2004	4	0.041	0.037
18	C:P1.5,2:F5–8	2	BY (1)	42,665	2004	4	0.001	0.006
19	C:P1.5,2:F3–6	2	BY (1)	148,953	2004	4	0.007	0.01
20	B:P1.5–1,2–2:F5–8	2	HE (1), NI (1)	3,076,129	2004	<1	0.002	0.002
21	B:P1.7–2,4:F5–1	2	BY (1)	243,545	2004	<1	0.003	0.003
22	B:P1.7–2,13–9:F5–5	2	NW (1)	239,183	2005	4	0.001	0.001
23	B:P1.7–2,16:F3–3	5	TH (3)	2,399,167	2005	24	0.001	0.001
24	B:P1.7–2,4:F1–5	10	NW (3), RP (1), SL (1)	1,524,166	2005	22	0.001	0.001
25	C:P1.22,14:F3–3	2	BB (1), SN (1)	1,512,043	2005	5	0.012	0.01
26	C:P1.5,2:F3–6	2	BY (2)	520,190	2005	7	0.018	0.02

*BY, Bavaria; NI, Lower Saxony; HH, Hamburg; TH, Thuringia; HE, Hesse; RP, Rhineland-Palatinate; SL, Saarland; NW, North-Rhine-Westphalia; BB, Brandenburg; SN, Saxony; BW, Baden-Wuerttemberg; MV, Mecklenburg-West-Pomerania.
†p values from the unadjusted 42-mo scan,
‡p values from the age-adjusted scan (p value >0.05 is shown in *italics*).
§FetA type 5-X has not yet been assigned.
¶Not detected because of missing date of birth in 1 case (see text).

automatically before the scan. The date of specimen sampling was defined as time of illness and the county of residence, derived from the postcode, was used as place. The date of submission to NRZM was used if the date of sampling could not be determined (in 2.5% of all cases). Spatiotemporal scanning was initiated at the centroids of the 439 German counties. These represent intermediate administrative units between the German states and the local levels (Gemeinden) and vary in size and population (there are 35,846–3,392,425 inhabitants/county). A county, which can also be a larger town, is the smallest public health unit. The maximum spatial cluster size was chosen to correspond to 7% of the German population (5,777,219). The maximal temporal cluster size was set to 30 days. Age-adjusted scans were performed with age groups >18 years of age and ≤18 years of age as a covariate. Adjustments for missing data were made according to the program's user manual to account for counties that did not refer samples to NRZM yet reported cases to the Robert Koch-Institute (the central federal German institution responsible for disease control and prevention). Clusters were considered significant for p values ≤0.05. Each finetype was analyzed separately.

Geographic Maps

Latitude and longitude coordinates (map date WSG 84) of the centroids of each German county and age-stratified census data of the year 2003 were obtained from GfK Macon (Waghäusel, Germany). Maps were generated and edited with the programs Regiograph 8 (GfK Macon) and Fireworks MX Macromedia (Adobe Systems Inc., San Jose, CA, USA).

Results

Finetyping of Meningococci

We analyzed data from 1,616 patients who contracted IMD in Germany from December 2001 through June 2005. Geographic data were inferred successfully from the 5-digit postcode for all patients. Complete finetyping results (serogroup: P1.VR1,VR2:FVR1) were available for all patients. A rank-abundance plot of all finetypes found during the study period indicated the diversity of pathogenic meningococci (Figure 1). The proportion of persons ≤18 years of age was 74.1%. The serogroup distribution was 65.7%, 28.8%, 3.1%, 1.9%, and 0.3% for serogroups B, C, Y, W135, and 29E, respectively. One case each was caused

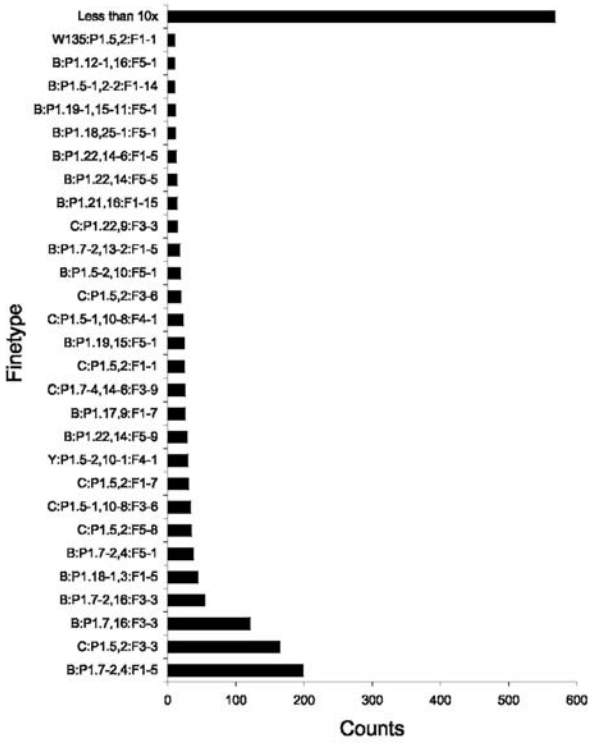

Figure 1. Distribution of 383 finetypes included in the present study (1,616 patients). The most common finetype (B:P1.7–2,4:F.1–5) accounted for 12.3% of the cases.

by serogroups A and Z and a capsule null locus isolate (*26*). A total of 33, 69, and 66 variants of PorA VR1, PorA VR2, and FetA, respectively, were identified. The number of unique combinations of serogroups PorA VR1, PorA VR2, and FetA VR was 383. FetA typing increased the number of finetypes 2.3-fold compared with the number of serogroup PorA VR1 and PorA VR2 combinations alone (167 distinct types). After removing all but 1 strain per cluster from the complete set of data, we determined the numeric index of the discriminatory ability of our typing procedure. Its value for serogroup:PorA typing was 0.930 (95% CI 0.923–0.937) compared with 0.963 (95% CI 0.959–0.968) for serogroup:PorA:FetA typing. The addition of FetA typing increased the discriminatory power of our typing procedure.

Cluster Analysis

SaTScan analysis was applied separately to each finetype present more than once in the historic dataset (134 finetypes) to identify clustering of meningococcal disease in space and time to a degree beyond that expected by chance alone (Table). Analysis identified 26 clusters that included 68 cases (4.2% of all cases). The maximum number of patients per cluster was 10. The median duration of the clusters was 4.0 days (range <1–24 days) and the median interval between the first and the second case was also 4.0 days (range <1–23 days). The median population within the scan windows imposed by SaTScan was 475,011 (range 42,665–5,441,714). In 76.9% of the clusters, only 2 patients were assigned to a cluster. Figure 2 shows the retrospective identification of a cluster of the finetype C:P1.5,2:F3–3 (cluster 10, Table).

Adjustment for Potential Confounders

The following potentially confounding variables were evaluated. Scans were adjusted for age because most cases occurred in persons ≤18 years of age (74.1%), the proportion of which was nonhomogeneously distributed per county (range 12%–26%, data not shown). The date of birth was missing for 8 patients (0.4%), who were therefore excluded from the adjusted scan. Only 1 cluster identified in the nonadjusted scan was assigned a p value >0.05 after age-adjustment (cluster 16, Table). Age did not substantially confound the results of the scan.

Underreporting to NRZM by some counties was addressed. For cases submitted in 2003, we performed SaTScan analysis excluding 66 counties identified as having cases of IMD reported to the Robert Koch-Institute but not to NRZM. The adjusted scan resulted in slight changes in the p values of detected clusters compared with the unadjusted scan but detected the same clusters (data not shown).

Discussion

Our study quantified the proportion of IMD cases that occurred in clusters in a large central European country in a period of 42 months. The following technical prerequisites permitted this large-scale investigation: free availability of the cluster detection software SaTScan, implementation of an automatic data transfer between our database and SaTScan, availability of data regarding time and place of occurrence of IMD, and state-of-the-art highly discriminatory finetyping techniques for the infectious agent in question.

The proportions of different antigenic profiles of meningococci represented by finetypes are subject to temporal and spatial changes because of constant interaction with host immunity (*27*). Compared with sequence types obtained by MLST, PorA and FetA finetypes are expected to fluctuate to a greater extent over time. The application of PorA and FetA finetyping for cluster analysis is warranted because of its consistency within clusters appearing for days or weeks. Exceptions exist, e.g., an outbreak of meningococci differing in its ability to express *porA* has been reported (*28*). However, this phenomenon would not affect cluster detection by our approach because we used sequence-based typing. We detected 1 epidemiologically

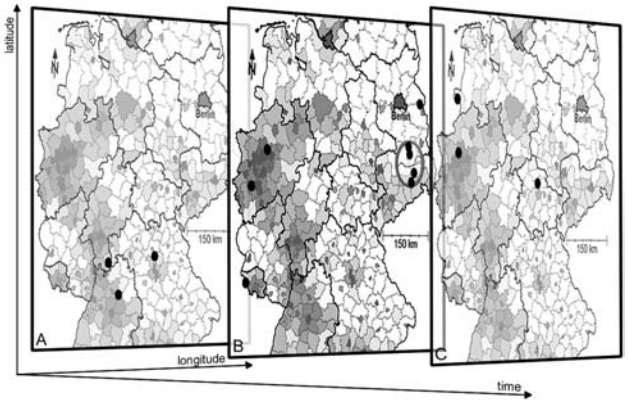

Figure 2. Retrospective identification of a cluster (cluster 10) of the finetype C:P1.5,2:F3-3 in 3 temporal planes using SaTScan (*11*). Planes A, B, and C represent consecutive temporal windows of 30 days in 2003. Cases with the finetype in question are shown by dark ovals defined by the dimensions longitude, latitude, and time. Although planes A and C do not show spatial clustering, plane B shows an accumulation of 4 cases in 2 counties within a circle encompassing a population of 339,185 (radius 28.78 km, p = 0.004; marked by a gray oval). Counties of Germany are shaded according to their population density (darker indicates a higher population density).

related cluster in which strains did not uniformly contain the *fetA* gene (cluster 11, Table). In general, we believe that these examples represent exceptions. MLST is probably not suitable for a timely national laboratory surveillance of clusters of meningococcal disease because of its considerable requirement for resources. The dataset reported here is the first comprehensive application of FetA typing, which was introduced as an alternative marker for meningococci in 2003 (*3*). FetA typing has proven to be reliable and easy to use. Moreover, it increased the discriminatory power of our typing procedure. The results of this study thus support the extended use of FetA sequence typing.

SaTScan was chosen because it is the most thoroughly evaluated software for detecting spatiotemporal clusters of infectious diseases. Application of a Poisson distribution to the epidemiology of a rare disease such as IMD is appropriate, although in practice the null hypothesis (i.e., complete spatial randomness) cannot be expected to be true even if no clusters of disease exist for a given spatiotemporal expanse. SaTScan serves as a tool that directs the attention of its user to anomalous case distributions. The p values are automatically adjusted for the multiple testing stemming from repeated evaluations of different potential clusters during hypothesis testing.

Hoebe et al. applied a global clustering test (space-time nearest neighbor analysis) to different serosubtypes of meningococci and found statistical evidence for clustering in 6 of 25 clusters reported by the Dutch Inspectorate of Health Care (*10*). However, connections of >2 cases could not be demonstrated beyond chance. Since only phenotypic typing was performed, the analysis was restrained by a considerable proportion of nontypeable isolates. In contrast to the Dutch study, we included both viable strains and sterile specimens in our investigation. Only fully-typed strains and DNA were evaluated for the existence of spatiotemporal clusters. Since we used a cluster detection test rather than a global clustering test, we were able to pinpoint clusters of meningococcal disease even for rare finetypes in space and time. The detection of the presented clusters as such did not depend on the attentiveness of public health workers. Spatiotemporal proximity could be shown for up to 10 patients (cluster 24, Table). Similar to findings of other studies (*29*), most clusters had only 2 patients.

The temporal settings of our scans were defined according to results from earlier retrospective cluster studies. An American analysis (*30*) found that 73% of secondary cases appeared ≤14 days after the index case. In France, 72% of secondary cases occurred in the first week after the first case (*5*). A British survey determined the median intervals between the index case and the second case to be 1.5, 5, or 23 days, depending on the setting of the cluster (household, school, or university) (*29*). Thus, a maximal temporal window of 30 days should detect most of the existing clusters, although the time between the first and the second case may rarely exceed this temporal limit.

Two spatially confined immunization campaigns were conducted after outbreaks of IMD caused by ET-15 meningococci. Our analysis detected 2 clusters representing each of them (clusters 11 and 19). The first campaign targeted a single county in North-Rhine-Westphalia; the second one comprised only a few boroughs within a county in Bavaria (*31*). Theoretically, spatially uneven vaccine coverage could introduce a regional bias into our analysis, e.g., by creating areas with low carriage rates (herd protection). Because of the low number and confined nature of the campaigns, a possible bias was not assessed but is likely negligible. A general recommendation for vaccination against serogroup C disease in Germany was made in 2006, i.e., after this study. Previously, only the State of Saxony had a general recommendation. However, reimbursement of costs was not guaranteed there, and precise numbers of vaccinees are not available. One also has to consider that most cases of meningococcal disease in Germany are not preventable by vaccination.

Most clusters of meningococcal disease occur in households or social units that provide educational services in workplaces, and through other forms of social interaction. To curtail computing time while providing an acceptable geographic resolution, counties represented the smallest

geographic units in our analysis. The variable size of the counties leads to fewer possible cluster locations evaluated in the area of large counties. The increased geographic aggregation in larger counties may also reduce the power to detect small clusters. However, since maximal spatial cluster size was chosen to correspond to 7% of the German population, detection of clusters spanning neighboring counties was warranted in all positions of our grid (e.g., Berlin's population plus that of the counties encircling it comprise <7% of the population of Germany). Performing cluster analyses on the basis of the patient's residence may not always reflect the area of the social network where acquisition of IMD occurred. Infection might be contracted at locations other than the one suggested by the postcode, e.g., at gatherings outside the county of residence. Thus, a few supraregional clusters might have been missed by our approach.

The proportion of patients involved in clusters in Germany was 4.2% (95% CI 3%–5.3%). Interpretation of this figure must consider that not all cases of IMD are assessed at NRZM. Conversely, all clusters reported herein were verified by finetyping. In 42 months, 26 clusters were detected. In France, 28 clusters of meningococcal disease were identified within 2 years, as shown by a household and institution survey. A total of 4.5% of all cases were either coprimary cases or secondary cases (*5*). Historic analysis comprising nearly 40 years based on Israeli health ministry investigations suggested that 13% of all cases were involved in outbreaks (*32*). In England and Wales, 0.5% of all cases investigated were secondary cases among close family and household contacts (*4*). Approximately 20 clusters occurred in England and Wales per year in preschool and school settings (*33*). Thus, epidemiologic surveys suggest that only a few cases are involved in clusters of IMD. This finding is supported by the results of our analysis.

The combined use of medical informatics and molecular laboratory techniques recently assisted detection of a methicillin-resistant *Staphylococcus aureus* outbreak in the hospital setting (*34*). The almost seamless integration of SaTScan into the database of NRZM will enable us to implement an early-warning system embedded in a geographic information system. This will support public health investigation of a serious community-acquired disease. We are currently evaluating the benefits of prospective cluster analyses and their immediate reporting to public health officials for management of IMD.

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Schistosomiasis among Travelers: New Aspects of an Old Disease

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Schistosomiasis is increasingly encountered among travelers returning from the tropics; signs and symptoms of travelers may differ from those of local populations. During 1993–2005, schistosomiasis was diagnosed in 137 Israeli travelers, most of whom were infected while in sub-Saharan Africa. Clinical findings compatible with acute schistosomiasis were recorded for 75 (66.4%) patients and included fever (71.3%), respiratory symptoms (42.9%), and cutaneous symptoms (45.2%). At time of physical examination, 42 patients (37.1%) still had symptoms of acute schistosomiasis, chronic schistosomiasis had developed in 23 (20.4%), and 48 (42.5%) were asymptomatic. Of patients who were initially asymptomatic, chronic schistosomiasis developed in 26%. Diagnosis was confirmed by serologic testing for 87.6% of patients, but schistosome ova were found in only 25.6%. We conclude that acute schistosomiasis is a major clinical problem among travelers, diagnostic and therapeutic options for acute schistosomiasis are limited, and asymptomatic travelers returning from schistosomiasis-endemic areas should be screened and treated.

Schistosomiasis is a common parasitic infection in the developing world, especially in Africa, where it is hyperendemic in many regions. In Israel, schistosomiasis is no longer endemic; however, cases have been reported in travelers returning to Israel. Research on the clinical, diagnostic, and therapeutic aspects of chronic infection in disease-endemic populations is extensive; however, data about schistosomiasis in travelers returning from these areas are relatively sparse. Moreover, because local inhabitants of disease-endemic areas are exposed repeatedly, the clinical manifestations may differ between residents and visitors. We examined the epidemiology and clinical manifestations of schistosomiasis in travelers returning to Israel.

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Methods

We used a list of all laboratory-confirmed diagnoses of schistosomiasis in Israelis from the Ministry of Health in Jerusalem, Israel, and the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA, during 1993–2005. Schistosomiasis is reportable in Israel, and all diagnoses of schistosome ova in stool or urine in patients should be referred to the central laboratory of the Ministry of Health. Before August 2003, serologic tests were mostly referred to CDC for fast-ELISA screening with *Schistosoma mansoni* adult microsomal antigen and for confirmation and speciation by enzyme Western blot, which has a sensitivity and specificity of ≈100% (*1*). Since then, serologic diagnosis of schistosomiasis has become available at the Ministry of Health and uses a soluble egg antigen ELISA (IVD Research Inc., Carlsbad, CA, USA) that is not species specific.

Clinical and epidemiologic data were available for all cases diagnosed and patients treated at the Center for Geographic and Tropical Medicine at Sheba Medical Center. If data were incomplete, patients were contacted by phone for additional details. We examined only cases in Israeli travelers; cases in immigrants from disease-endemic countries, who may have been previously exposed to *Schistosoma,* and foreign workers were excluded. The study was approved by the ethical committee of Sheba Medical Center.

Data were analyzed according to the presence of symptoms suggestive of acute schistosomiasis, the evolution to chronic schistosomiasis (genital, urinary, or gastrointestinal), or the diagnosis of asymptomatic schistosomiasis through screening. A case of acute schistosomiasis was defined as the onset of fever or hypersensitivity symptoms—urticarial rash, angioedema, dry cough or wheeze—after exposure to infected water sources, with subsequent confirmation by serologic or parasitologic testing. A source of infection was defined as self-reported exposure to fresh water (e.g., bathing, diving) in a schistosomiasis-endemic

region. Exposure time was measured from the first day of exposure to the last. The time from exposure to illness was measured from the last exposure to the onset of symptoms. The Fisher exact test and Student *t* test were used to analyze qualitative and quantitative variables, respectively; level of significance was set at $p<0.05$.

Results

During 1993–2005, a total of 137 travelers returning to Israel had a laboratory diagnosis of schistosomiasis. The mean annual number of cases was 10.5 (range 1–13 cases/year) with the exception of 1998, when 27 cases associated with rafting trips to the Omo River in Ethiopia were diagnosed (*2*). The mean ± SD age of the travelers was 27.1 ± 6.8 years, and the male:female ratio was 2.42:1. *S. haematobium* caused 39.4% of cases; *S. mansoni* caused 29.9%, a mixed infection caused 16%, and undetermined species (because the test was not species specific) caused 14.7%. One patient was seropositive for *S. mekongi*.

Epidemiologic data were available for 113 (82.5%) patients. All infections were acquired in Africa except 1, which was acquired by a traveler who had bathed in the Mekong River in East Asia. Twenty-five (22.1%) patients were exposed to 2 potentially infected water sources. The other patients were exposed to only 1 source, mostly Lake Malawi (57%) and the Omo River in Ethiopia (18%). The median exposure time was 2 weeks (interquartile range 1.5–3.0 weeks).

Clinical data were available for 113 patients, of whom 104 were evaluated at the Sheba Medical Center (Table 1). Of these, 48 (42.5%) were asymptomatic at the time of evaluation, and the rest had symptoms of either acute or chronic schistosomiasis (Figure); demographics and duration of travel were similar regardless of the symptom phase at evaluation (Table 1). Immediate symptoms after bathing (acute itching and rash, suggestive of swimmer's itch) were reported by only 8 (7.1%) patients.

Acute Schistosomiasis

Of the 42 (37.2%) patients with acute schistosomiasis, symptoms occurred after a median of 3 weeks (mean ± SD 3.1 ± 2.7 weeks) from exposure. Fever was the prevalent symptom, followed by respiratory symptoms and rash. Only 4 (9.5%) patients had the complete complex of acute schistosomiasis: fever, urticaria, and respiratory symptoms. An additional 33 patients reported having had an episode of symptoms after exposure that was compatible with acute schistosomiasis but resolved before medical examination (Table 2). For most patients, the duration of fever and urticaria was short, usually 1–2 weeks (range 1–6 weeks), and the median duration of respiratory symptoms was longer, 6 weeks (mean ± SD 15.4 ± 22.7 weeks).

Chronic Schistosomiasis

Of the 23 (20.3%) patients with chronic schistosomiasis, time from exposure to examination ranged from 4 months to 3 years (mean ± SD 58.0 ± 31.5 weeks). Among them, 13 had a history of an acute schistosomiasis-like illness, 21 (91.3%) had genitourinary symptoms (17 hematuria or dysuria, 4 hematospermia), 2 had gastrointestinal symptoms, and 1 had protracted fatigue and abdominal pain. No patient had renal failure, obstructive uropathy, cirrhosis, or central nervous system involvement; however, 7 (30.4%) patients underwent invasive diagnostic procedures for suspected tumors of the bladder or colon, and their travel history was considered only after the diagnosis of chronic schistosomiasis had been made.

Asymptomatic Schistosomiasis

A total of 48 (42.5%) patients were examined while asymptomatic. These patients included 5 who were referred because of eosinophilia and 5 who were hospitalized in Israel because of malaria and had serologic testing because of their travel history. The rest of the patients came in for screening because schistosomiasis had been diagnosed in a traveling companion or because they had been exposed to fresh water while traveling. A history of

Table 1. Clinical and laboratory data for patients with schistosomiasis, by stage of disease, Israel, 1993–2005

	Acute (n = 42)	Chronic (n = 23)	Asymptomatic (n = 48)	p value
Age ± SD, y	28.1 ± 8.0	25.1 ± 2.6	27.2 ± 6.8	NS*
Male:female ratio	2.63:1	4.5:1	2.5:1	NS
Exposure, weeks ± SD	2.3 ± 1.9	4 ± 5.8	2.1 ± 0.8	NS
Exposure to symptoms, weeks ± SD	3.1 ± 2.7	58.0 ± 31.5	–	<0.001†
Eosinophil count, $\times 10^9$/L ± SD	2,374 ± 1,937	864 ± 529	1,363 ± 1,490	<0.05† NS‡
Schistosoma haematobium§	42.5%	60.9%	27.1%	NS
S. mansoni§	27.5%	0%	52.1%	<0.05†‡
Ova detection	25%	56.5%	14.6%	<0.02† NS‡
Serologic diagnosis	95%	96%	89.5%	NS

*NS, not significant.
†Comparison between acute and chronic schistosomiasis.
‡Comparison between acute and asymptomatic schistosomiasis.
§In 14.7% of all cases, the test used was not species specific.

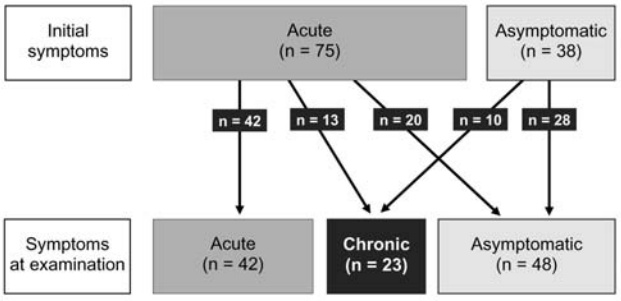

Figure. Progression of schistosomiasis symptoms among Israeli travelers (n = 113), 1993–2005.

an acute schistosomiasis-like illness was elicited from 20 of these asymptomatic patients.

Laboratory Findings

For patients with acute schistosomiasis, physical examination findings were usually unremarkable. Laboratory test results showed eosinophilia in 73% and mildly abnormal liver function in 17%. Eosinophilia was more prevalent in patients with acute than with chronic schistosomiasis (Table 1).

Diagnoses were confirmed by serologic testing for 120 (87.6%) patients. For only 35 (26.9%) patients, mostly those with chronic schistosomiasis, were schistosome ova found in urine, semen, or stool samples (Table 1). For 6 patients, the diagnosis was established by chance in a tissue biopsy; subsequent testing showed ova in the stool or urine of 5 of these 6 patients. A diagnosis of schistosomiasis was made for 1 additional patient, whose colectomy specimen (colectomy performed because of familial adenomatous polyposis) contained schistosome ova 10 years after exposure (*3*).

Treatment

Praziquantel was offered to all 113 patients, but follow-up information after treatment was available for only 65. Eosinophil counts declined from a mean ± SD of $2,100 \times 10^9$/L ± 1,850 to 970×10^9/L ± 1,575 ($p<0.01$). Because respiratory symptoms were the least self-limiting form of acute schistosomiasis, response to therapy was evaluated in these patients. Among the 38 patients with respiratory symptoms, 4 cases resolved before praziquantel therapy, and follow up was incomplete in 12. Of the remaining 22 patients, in 9 cases there was an acute exacerbation of symptoms after praziquantel therapy, lasting from a few days to 3 weeks. Corticosteroids were prescribed for 5 patients with protracted respiratory symptoms, and repeated courses of praziquantel were prescribed for 6. Overall, in 20 of 22 cases, symptoms improved within 2 months after therapy. A history of asthma was not associated with a worsening of symptoms after therapy.

Discussion

Although schistosomiasis is widespread throughout much of the tropics, we found that among Israeli travelers, schistosomiasis is acquired almost exclusively in Africa. This finding supports data from the GeoSentinel group and the TropNetEurope surveillance network, which report that Africa was the source for ≈90% of all cases (*4*,*5*). The absence of cases from other disease-endemic regions may be because schistosomiasis is often a focal disease, and perhaps infections are not commonly found in the specific locations frequented by travelers in South America and the Far East.

The schistosome life cycle has 3 phases in the human host, and all have their clinical counterparts. The first phase is host penetration by cercaria, which is usually manifested as a transient (hours) itchy eruption that occurs soon after exposure and is known as swimmer's itch or cercarial dermatitis. The second phase is schistosomulae tissue migration and maturation and is associated with transient (days to weeks) hypersensitivity, which gives rise to the syndrome of acute schistosomiasis. The third phase, endovascular egg production, is protracted (years) and is associated with organ damage (genitourinary, gastrointestinal, or ectopic migration to other organs).

In our cohort, a history suggestive of the first phase, swimmer's itch, was infrequent, found in 7.1% of patients. Other case series also suggest that it occurs in only 12%–36% of patients with schistosomiasis (*6*–*8*). For many of our patients, data were collected long after exposure, so

Table 2. Symptoms reported by patients with acute schistosomiasis, Israel, 1993–2005

Symptom	During examination (n = 42), no. (%)	During examination and by history (n = 75), no. (%)
Fever	30 (71.3)	51 (68.0)
Respiratory*	18 (42.9)	30 (45.0)
Cutaneous†	19 (45.2)	23 (30.7)
Combinations		
Fever and respiratory	6 (14.2)	11 (14.7)
Fever and cutaneous	11 (26.2)	13 (17.3)
Fever, cutaneous, and respiratory	4 (9.5)	5 (6.7)

*Cough, wheeze, dyspnea.
†Pruritus, urticaria, angioedema.

they may have forgotten such a transient phenomenon. Obviously, the absence of a history of swimmer's itch cannot be used to exclude schistosomiasis.

Our data show acute schistosomiasis, the second phase, to be the predominant form of the disease in travelers, noted by 66.4%. Travelers in that respect are markedly different from local populations and immigrants, among whom acute schistosomiasis is rare. Some textbooks suggest that acute schistosomiasis is a syndrome that typically results from heavy infestations with *S. japonicum* or *S. mansoni* and rarely, if ever, from *S. haematobium* (*9*). Our findings suggest that, to the contrary, acute schistosomiasis occurs with *S. haematobium* and with *S. mansoni*. We believe that acute schistosomiasis is an immune phenomenon, is not species specific, and can develop after infection with each of the schistosomae that infect humans. That some species (e.g., *S. mekongi* and *S. interacalatum*) are rarely reported in the literature as causing acute schistosomiasis may merely reflect lack of traveler exposure.

The acute symptom complex that appears several weeks after exposure is often called Katayama fever, having been described in Katayama district near Hiroshima in Japan in 1847 (*10*). However, our data show that fever occurs in only 71% of patients with acute schistosomiasis; similarly, data from the early 20th century show that fever was not universally present during the acute toxemic phase (*11*). We therefore believe that acute schistosomiasis, rather than Katayama fever, should be the preferred name for this syndrome.

Although fever is indeed not universal with acute schistosomiasis, when it does occur, it is usually high; therefore, malaria is a major differential diagnosis. This may help explain why most patients evaluated abroad were treated for malaria. Antimalarial drugs were offered to some patients despite negative smear results. In other patients reported to have positive malaria smear results in Africa, serologic testing for plasmodia after their return was found to be negative, which adds to reports of the unreliability of tests performed in Africa (*12*). For other patients, fever and urticarial rash were diagnosed as allergic reactions and treated with glucocorticoids. Urticarial rash or eosinophilia are clinical clues that may help differentiate between the fever of acute schistosomiasis and that of malaria. Also, persistent fever despite documented parasitologic response in cases of proven malaria should alert the clinician to the possibility of coinfection.

The 3 major clinical features—fever, skin, and respiratory symptoms—occur in combination in only a small percentage of patients with acute schistosomiasis (Table 2). Even then, they often do not appear simultaneously. Unfortunately, in our experience, even when returning travelers had a history of the complete symptom complex, the clinical diagnosis was frequently missed. The respiratory symptoms tend to follow a more protracted course, and unless a thorough history is taken, the case may be misdiagnosed as allergy or asthma.

In chronic schistosomiasis, the third phase, genitourinary problems and, less often, gastrointestinal problems occur. Late sequelae such as cirrhosis, portal/pulmonary hypertension, or obstructive uropathy are the aftermath of heavy or repeated infection typical for local populations; absence of these sequelae among travelers is not surprising. Of seropositive patients who were initially asymptomatic, chronic schistosomiasis developed in 26% (Figure). The question of whether asymptomatic travelers with history of freshwater exposure should be screened and treated for schistosomiasis is clearly pertinent (*2*). Our data strongly support the recommendation to screen and treat.

Our study illustrates the problems encountered in diagnosing *Schistosoma* infection in patients evaluated during acute schistosomiasis. Stool and urine examinations for ova were largely negative, diagnoses relied on serologic testing, and therefore most diagnoses were made post facto. Although serologic testing appears to be very sensitive, a caveat must be added that most serum samples were collected relatively late in the course of acute schistosomiasis. For the acutely ill febrile returning traveler, serologic test results may be negative, as occurred for 2 of our patients for whom an additional specimen was required for diagnosis.

The ability to diagnose chronic schistosomiasis by ova detection was higher (Table 1); however, for many patients, only serologic testing gave a positive result. Thus, ova detection, which is the main diagnostic tool in schistosomiasis-endemic countries, often gives negative results for travelers. Negative results occur during the acute stage because symptoms start before oviposition and during the chronic stage because of low-level or intermittent oviposition. Successful ova detection entails multiple examinations of stool or urine; sensitivity depends greatly on technique and expertise, which may be declining at many Western institutions.

Treatment of schistosomiasis can also be problematic. Praziquantel is considered the drug of choice for schistosomiasis but possesses little activity against the juvenile forms of the helminth (schistosomulae) (*13*). Of our patients who were exposed in Lake Malawi, 11 took praziquantel soon after exposure while abroad, as a form of preemptive therapy for later schistosomiasis, which is a common recommendation for visitors to Lake Malawi. Of these, acute schistosomiasis developed in 7, symptoms lasted 5–45 weeks, and chronic schistosomiasis developed in 2. These cases show that praziquantel given shortly after exposure does not preclude later clinical infection.

The limitations of praziquantel for treating acute schistosomiasis were clearly seen in our patients. For those who

had fever and rash, symptom duration was relatively short, and the contribution of therapy is unclear. For patients with respiratory symptoms, therapy was sometimes associated with an acute exacerbation of symptoms. For some patients, cough and dyspnea continued for many months after treatment. Our policy is to repeat praziquantel treatment after 3 months, which may be why we did not see chronic schistosomiasis in this group.

Conclusions

Most cases of schistosomiasis in Israeli travelers are acquired in sub-Saharan Africa. For travelers, most symptomatic cases of schistosomiasis are acute, diagnosis by ova detection is usually negative, and serologic testing, when performed too early, may give false-negative results. Modern practitioners may not be in a better situation than those who treated acute schistosomiasis cases in the 1920s (*11*). Our findings lend support for screening asymptomatic travelers to schistosomiasis-endemic areas because chronic schistosomiasis will develop in many of them. Better knowledge of the various possible manifestations of schistosomiasis should decrease the time to diagnosis of acute and chronic schistosomiasis. Physicians should promptly evaluate returning travelers for schistosomiasis even if they do not report the complete symptom complex. However, in the absence of effective treatment for acute schistosomiasis, new diagnostic methods and new drugs that affect the parasite at an early stage are needed.

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Gastroenteritis and Transmission of *Helicobacter pylori* Infection in Households[1]

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The mode of transmission of *Helicobacter pylori* infection is poorly characterized. In northern California, 2,752 household members were tested for *H. pylori* infection in serum or stool at a baseline visit and 3 months later. Among 1,752 person considered uninfected at baseline, 30 new infections (7 definite, 7 probable, and 16 possible) occurred, for an annual incidence of 7% overall and 21% in children <2 years of age. Exposure to an infected household member with gastroenteritis was associated with a 4.8-fold (95% confidence interval [CI] 1.4–17.1) increased risk for definite or probable new infection, with vomiting a greater risk factor (adjusted odds ratio [AOR] 6.3, CI 1.6–24.5) than diarrhea only (AOR 3.0, p = 0.65). Of probable or definite new infections, 75% were attributable to exposure to an infected person with gastroenteritis. Exposure to an *H. pylori*–infected person with gastroenteritis, particularly vomiting, markedly increased risk for new infection.

Helicobacter pylori infects at least 50% of the world's population. Infection occurs in early life (*1*,*2*). Because acute infection invariably passes undetected, however, the precise age of acquisition is unknown. In industrialized countries, infection rates are declining rapidly (*1*,*3*), but high rates of infection persist among disadvantaged and immigrant populations (*4*,*5*).

The mechanisms of *H. pylori* transmission are incompletely characterized. Person-to-person transmission is most commonly implicated with fecal/oral, oral/oral, or gastric/oral pathways (*6*); each has supportive biologic as well as epidemiologic evidence. Like many common gastrointestinal infections, infection is associated with conditions of crowding and poor hygiene (*7*,*8*) and with intrafamilial clustering (*9*–*12*). The organism has been recovered most reliably from vomitus and from stools during rapid gastrointestinal transit (*13*). These findings raise the hypothesis that gastroenteritis episodes provide the opportunity for *H. pylori* transmission.

Household transmission of gastroenteritis is common in the United States, particularly in homes with small children (*14*). If *H. pylori* is transmitted person to person, one might expect rates of new infection to be elevated after exposure to persons with *H. pylori*–infected cases of gastroenteritis. To explore whether diarrhea or vomiting contributes to disease transmission, we monitored northern Californian households experiencing gastroenteritis for new *H. pylori* infections and evaluated the contributions of household *H. pylori* infection and gastroenteritis to new infection. We were also interested in whether symptoms of new infection could be identified.

Methods

Population and Study Design

The study population consisted of households that were participating in the Stanford Infection and Family Transmission study, initiated in 1999 to prospectively evaluate the association of *H. pylori* infection and household gastroenteritis (*14*,*15*). The cohort, predominately Hispanic immigrant families residing in South San Francisco Bay, has a high seroprevalence of *H. pylori* infection (*16*,*17*). From January 2000 to July 2004, a total of 3,846 persons seen at 1 of 15 community (predominately low-

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income public health) clinics with diarrhea, vomiting, or both, were asked for permission to be contacted by a study representative for a phone screening. Of 2,941 households that could be contacted, 2,155 (73%) were eligible multiperson households (within 50 miles of the research site, within 12 days of gastroenteritis onset of index patients). Of these, 852 (40%) declined interest, and 1,303 (60%) were scheduled for a home visit. At the first home visit, consenting household members were interviewed regarding symptoms, onset, and duration of gastroenteritis within the previous 21 days. Blood and stool samples were collected for *H. pylori* testing.

Stool samples were requested from children <2 years of age and from others who refused phlebotomy. Approximately 3 months later (range 12–20 weeks), household members were reinterviewed, and a second specimen was collected for *H. pylori* testing. Participation was voluntary; small gifts (i.e., a mug, a tote bag, hand antiseptic, a calendar) were offered as thanks for participation. The study was approved by the institutional review boards of Stanford University, Santa Clara and San Mateo Counties, and the state of California.

Laboratory Methods

H. pylori Serologic Testing

Anti–*H.pylori* immunoglobulin G (IgG) was quantified by using an in-house ELISA (*18*), previously validated and adapted for use in US, Latin American, and Asian populations (*19*–*21*). Optical density (OD) results were categorized as negative (<85% of low positive standard), borderline (85%–110% of low positive standard), or positive (≥110% of low positive standard). In different series of control samples with biopsy-confirmed infection, the assay, which includes 6 different isolates (2 from Mexico), is 91% sensitive and 98% specific for infection in adults (*15*,*18*). For our study, an equivalent sensitivity and specificity were established for children >2 years of age by using samples from controls with biopsy-confirmed infection from a separate study to establish a lower OD cut-off (75% of adult control) (*15*). Serologic testing results are considered unreliable in children <2 years of age (*22*).

Each serum sample was tested in triplicate for *H. pylori* on 2 occasions, soon after it was received and later, when it was paired with the second visit sample from the same study participant. Between testing, samples were frozen at −80°C. The paired serum results are presented here. High reproducibility between first and second runs of the same sample (mean coefficient of variation 18 ± 17) suggests that the effect of freezing or storage was negligible. Titer levels were derived from ODs by standard curve methods. A seroconversion was defined as a qualitative change from negative to positive, negative to borderline, or borderline to positive, if accompanied by ≥4-fold increase in *H. pylori* titer from baseline. A seroreversion was defined as a qualitative change from positive to negative, accompanied by ≥2-fold decrease in *H. pylori* titer.

To corroborate recent *H. pylori* infection (*23*), serum samples from 22 IgG seroconverters and 22 randomly sampled, persistently seronegative adults were tested for *H. pylori* IgM antibody response in the laboratory of Dr. Perez-Perez by using a mixed strain assay previously validated in ethnically diverse and pediatric populations (*9*,*24*). Detection of an IgM antibody response at either first or 12-week follow-up visit was considered a positive test result.

H. pylori Stool Antigen

Stool antigen was tested with the Premier Platinum HpSA enzyme immunoassay (Meridian, Cincinnati, OH, USA). Stool samples collected at home visits were transported to the laboratory and stored at –20°C until processed. Samples not available at the home visit were expressed by overnight mail. In 1 metaanalysis, stool antigen had a mean sensitivity and specificity of 91% and 93%, respectively (*25*); however, accuracy may be lower in children <6 years of age (*26*). In the present study population, *H. pylori* was identified by PCR in 12 (46%) of 26 transiently positive stool samples from toddlers (vs. 0% of 10 stool antigen–negative samples), a finding that was consistent with the 50% sensitivity of PCR observed in *H. pylori*–inoculated stools (*27*). A stool conversion was defined as a qualitative change from negative to positive when the manufacturer's suggested cut-off values were used.

Testing Protocol

We requested stool and serum from all participants >2 years of age, although participants were included in the study if they offered 1 or the other. For practical reasons (i.e., stool samples are not typically available on demand and are unpleasant to ship) most persons >2 years of age (97%) provided only blood samples. Approximately 29% of children <2 years of age provided only blood samples, and 39% provided both blood and stool samples. The ^{13}C urea breath test was not considered because of costs and the accuracy of the applied methods (*22*).

Definitions

Gastroenteritis

Gastroenteritis was defined as either 1) ≥3 loose or watery stools per day in persons at least 2 years of age, or ≥5 per day in children <2, or 2) any vomiting. Symptoms lasting >14 days or reported symptoms due to potentially noninfectious causes (poisoning, pregnancy, chemotherapy) were excluded from the case definition (*14*,*15*).

Gastroenteritis was categorized as vomiting with or without diarrhea, or diarrhea alone. Only cases of gastroenteritis identified at the baseline visit were included in the present analysis.

New Infection

Given low expected rates of new infection and the role of testing error (*28*,*29*), we developed criteria for definite, probable, and possible infection on the basis of corroborative test results. A definite new infection was a seroconversion with confirmatory stool or IgM result. A probable new infection was a borderline seroconversion with confirmatory stool or IgM or a stool conversion with confirmatory IgG or IgM. Possible new infections met criteria for serologic or stool conversion without confirmation. Since serum specimens were not routinely obtained from children <2 years of age, a single stool conversion was the only way of diagnosing new infection in most of these children.

Persistent Negative/Persistent Positive

A persistent negative infection had no positive or equivocal test results during observation. A persistent positive infection had no negative or equivocal test results during observation. A transient infection was defined as a qualitative seroreversion with ≥2-fold decrease in *H. pylori* titer or a qualitative (positive to negative) stool reversion. Study participants with equivocal (40 qualitative seroconversions with <4-fold increase in *H. pylori* titer and 32 qualitative seroreversions with <2-fold decrease in *H. pylori* titer) or transient (7 serology and 28 stool) test results were not considered for outcomes but were included as baseline household exposures.

Household Exposure

A person was considered exposed to gastroenteritis, *H. pylori* infection, or *H. pylori* infection with gastroenteritis if ≥1 other person in the home had 1 of these profiles at the baseline visit. Household members who were not available for second visit testing (499 or 15% of baseline participants) were counted as baseline exposures but could not be considered for outcomes.

Statistical Analysis

Incidence of New Infection

We estimated the annual incidence of new infection and 95% confidence intervals (CIs) (*30*) for all ages and for age categories 0–2, 2–17, and ≥18 years. The outcome was the proportion of new infections in the age category, and the denominator was the total number of study participants in the age category who tested negative at baseline and who completed the second visit testing. For comparison, annualized rates of seroconversion and seroreversion are also reported. The denominator for estimating seroreversions included all persons who tested positive at the first visit and who completed second-visit testing.

Classification and Symptoms of New Infections

Classification of new infections (definite, probable, or possible) is described. The χ^2 test or Wilcoxon test for categorical and continuous measures, respectively, and logistic regression for multivariable adjustment were used to evaluate symptoms and other characteristics of new infections versus persistent negative results.

Incidence of New Infection by Household Exposure

We compared rates of new infection for study participants who resided with no known *H. pylori*–infected contact, with ≥1 infected contact, and with ≥1 infected contact who had gastroenteritis. The denominator for these estimates was the number of study participants within each exposure category who tested negative at baseline and who completed second-visit testing. Household members who were not available for second-visit testing or who completed both visits with equivocal changes in titer were counted as baseline exposures but could not be considered for outcomes.

Exposure to Gastroenteritis in an *H. pylori*–Infected Contact

Among study participants residing with ≥1 *H. pylori*–infected contact, we estimated the odds (and 95% CIs) of new infection, given exposure to infection with versus without gastroenteritis, including symptoms of gastroenteritis (vomiting versus diarrhea only). To minimize misclassification, the outcome for this analysis was restricted to definite and probable new infections, and the reference group with persistent negative results who had been exposed to *H. pylori* infection at baseline. A random intercept logistic regression model (Proc Glimmix., SAS/Stat, 9.2 ed., SAS Institute, Cary, NC, USA) was used to model household clusters and to adjust for age, sleeping density, and proportion of household members completing both visits, each modeled as a continuous variable. We also calculated the attributable risk (*30*) of new infection associated with exposure to *H. pylori* infection and to exposure to *H. pylori* infection with gastroenteritis. As a secondary analysis, we assessed risk factors for all new infections, including possible new infections.

Results

From January 2000 to June 2004, a total of 1,186 households were enrolled. These 1,186 households included 6,620 participants who participated in the first gastroenteritis interview, and 4,334 (65%) who also gave specimens. Households were predominately Spanish-

speaking and of low income (Table 1). Nearly three quarters (72%) of households had ≥1 *H. pylori*–infected household participant at the first visit.

Of enrolled households, 277 (23%), including 108 that dropped out and 169 that could not be located, did not complete the second visit (Table 1). Although the 909 households that did complete the study appeared somewhat smaller (median 5 vs. 6 household members, p = 0.06 Wilcoxon), the proportion of large households (>8 contacts) was not significantly different (20% vs. 19%). Conversely, enrollments (median 3 per household) were similar, although completing households were somewhat more likely to enroll more than the minimum of 2 participants (72% vs. 64%). Households that did not complete the study were also more likely to have been referred through an emergency department (21% vs. 15%, p = 0.02) but were similar in number of children enrolled, primary household language, sleeping density, educational attainment, and prevalence of *H. pylori* infection.

Incidence of New Infection

The 909 households that completed the second visit had 3,380 household participants; 2,881 (85%) completed both specimen collections. Of these, 129 (4.4%) were children <2 years of age who contributed only serum and were excluded from analysis because of lack of validation of our ELISA results in early childhood. Thus, a total of 2,752 household members, 2,372 (86%) with serologic results and 380 (14%) with stool or stool and serologic results, completed second-visit testing. Of these, 1,752 (64%) tested negative at baseline. Over a median 13 (±2) weeks of follow-up, 30 (1.7%) of these met the definition of a new infection, for an overall annualized incidence of 6.8% (95% CI 4.6%–9.8%). By serology, corresponding annualized seroconversion and seroreversion rates were 3.7% and 3.1%, respectively. Half of all new infections occurred in children <2 years of age, for an annualized rate of 20.9% (95% CI 11.8%–33.9%) in this age group versus 5.3% (2.4%–9.6%) in persons 2–17 years, and 3% (0.9%–6.5%) in persons ≥18 years.

Classification and Symptoms of New Infections

Among the 30 new infections, 7 met criteria for a definite new infection, 7 for a probable new infection, and 16 for a possible new infection (Table 2). The 7 definite IgG seroconversions included 4 corroborated with IgM and 3 seroconversions corroborated with change in stool antigen. Of the 7 probable new infections, 2 stool conversions (both in children <2 years of age) were corroborated with IgM, 4 borderline seroconversions were corroborated with IgM, and 1 borderline seroconversion was corroborated with stool antigen. Overall, 10 (45%) of the 22 stool or seroconversions tested had a positive IgM response at the first or second visit of the study participant, compared to 4 (18%) of 22 randomly selected persistent seronegatives (p = 0.05). The 16 possible new infections included 12 stool conversions, all in children <2 years, and 4 uncorroborated borderline seroconversions.

Overall, 175 children <2 years had serologic and stool results for each visit. Among 350 stool serum pairs, 335 (96%) were concordant, including 3 of 10 new infections and 2 transient infections. Of the 15 discordant results, 7 were stool conversions discordant at the study participant's second visit, 6 were stool reversions discordant at the participant's first visit (4 of these were corroborated by PCR in another study [*27*], and 2 were persistently stool positive or negative with discordant serology at 1 or both study participant visits).

Compared with 1,722 persistently negative results, the 30 new infections were in significantly younger study participants (median age 2 vs. 11 years, p<0.001) but of similar gender (40% male vs. 43% male, p = 0.78). When results were adjusted for age, new infections were somewhat more likely than persistently negative results to be in persons with gastroenteritis (adjusted odds ratio [AOR] 2.5, CI 0.97–6.6, p = 0.06), and the 14 definite or probable

Table 1. Household characteristics

Characteristics	Enrolled households (n = 1,186)	Households completing follow-up (n = 909)	Households not completing follow-up (n = 277)	p value
No. contacts, median (range)	6 (2–21)	5 (2–21)	6 (2–19)	0.06
No. enrolled, median (range)	3 (2–17)	3 (2–17)	3 (2–10)	0.03
No. children enrolled, median no. <18 y (range)	2 (0–11)	2 (0–11)	2 (0–9)	0.95
Educational attainment, median highest year (range)	12	12	12	0.42
% Spanish-speaking	72	73	71	0.65
Sleeping density (median persons/bedroom)	2.3/bedroom	2.5/bedroom	2.3/bedroom	0.66
Income (%<US$30,000/y)	59	58	62	0.39
≥1 *Heliobacter pylori* infected (%)	72	72	70	0.42
Emergency dept. referral (%)	16	15	21	0.02

Table 2. Case listing of 30 new infections*

Household–contact no.	Age (y)	GE	Criteria			No. *Helicobacter pylori*–infected contacts		
			IgG	Stool	IgM	Without GE	With diarrhea	With vomiting
Definite								
1–1	1.2	D/V	+	+	–	1	0	0
2–1	1.3	D/V	+	+	–	2	0	0
3–1	1.5	D/V	+	+	–	2	1	0
4–3	10	D/V	+		+	0	0	2
5–5†	11	D/V	+		+	0	3	1
6–3	23	D	+		+	0	0	1
7–2	37	D	+		+	3	0	1
Probable								
8–1	0.74	D/V		+	+	1	0	0
9–1	0.77	V		+	+	0	0	0
10–1	2.3	D/V	+ (B)	+		0	0	0
11–1	3.6	D/V	+ (B)		+	0	1	0
12–2†	21		+ (B)		+	2	0	0
13–2	23	D	+ (B)		+	0	0	1
14–2	42		+ (B)		+	0	0	0
Possible								
15–5	0.3			+		0	0	0
16–1	0.33	D		+	–	1	0	0
17–1	0.6	D/V		+	–	4	0	0
18–1	0.8	D/V		+		0	0	0
19–1	0.9	D/V		+	–	1	0	0
20–1	1.0	D		+	–	1	0	0
21–1	1.1	D/V		+	–	1	0	0
22–1	1.2	D/V		+		0	1	0
23–1	1.3	D/V		+	–	0	0	0
24–1	1.9	D/V		+		3	0	0
25–1	6.3	D		+		0	0	0
26–3	7		+ (B)		–	1	0	1
5–3†	7.6	D/V	+ (B)		–	0	3	1
28–1	7.8	V	+ (B)		–	3	0	0
29–4	8.5			+		2	0	0
12–4†	12.2		+ (B)		–	0	0	0

*GE, gastroenteritis; D, diarrhea only; V, vomiting only; D/V, diarrhea with vomiting; B, borderline seroconversion with ≥4-fold increase *in H. pylori* titer
†Cases occurring within the same household.

new infections were nearly 5 times more likely (AOR 4.9, CI 1.1–22.4, p = 0.04). No specific gastroenteritis symptoms for new infection were identifiable.

Incidence of New Infection by Household Exposure

Seven (*7*) new infections occurred in homes with no known *H. pylori*–infected participants (Figure) and 23 (77%) in homes with ≥1 infected contact, for rates of new infection of 1.1% and 2.1%, respectively (p = 0.10). Two households with exposure to an *H. pylori*–infected contact manifested 2 new infections (Table 2). Conversely, 1,319 (75%) study participants were exposed to gastroenteritis in another household member; new infection developed in 16 (1.2%), compared with 14 (3.2%) of 433 study participants not exposed (p = 0.005). However, new infection developed in 10 (2.9%) of 350 study participants exposed to an *H. pylori*–infected contact with gastroenteritis, compared with 6 (0.6%) of 969 of study participants exposed to gastroenteritis in an uninfected contact (p = 0.001).

Exposure to Gastroenteritis in an *H. pylori*–Infected Contact

Of 14 definite or probable new infections, 11 (79%) occurred in homes with ≥1 *H. pylori*–infected contact (Table 2). When adjusted for age, sleeping density, and proportion of household contacts completing both visits, exposure to an *H. pylori*–infected person with gastroenteritis increased risk for definite or probable new infection 4.8-fold (95% CI 1.4–17.1, p = 0.01) compared with exposure to infected persons without gastroenteritis. Exposure to an *H. pylori*–infected person with vomiting was a significantly stronger risk factor for new infection (p = 0.03) than exposure to an *H. pylori*–infected person with gastroenteritis but no vomiting (Table 3). The proportions of definite or probable new infections attributable to exposure to an *H. pylori*–infected person without and with gastroenteritis were 55% and 75%, respectively. Including possible new infections in the analysis decreased the magnitude of the associations with gastroenteritis (Table 3)

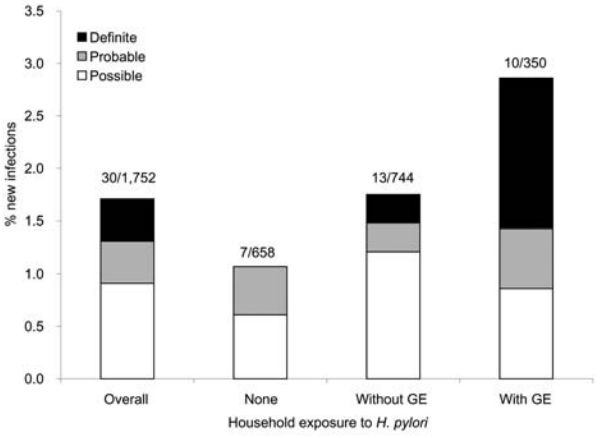

Figure. Rates of new *Helicobacter pylori* infection overall, without exposure to an infected contact (none); to ≥1 infected contact without gastroenteritis (Without GE), or to >1 infected contact who had gastroenteritis (With GE). Bar annotations denote number of new infections and number at risk. Definite/probable/possible, see text for classification of new infections.

although exposure to an *H. pylori*–infected person with vomiting remained a significant risk factor.

Discussion

In this prospective study of *H. pylori* infection and household gastroenteritis within a US immigrant population, we estimated an annualized *H. pylori* incidence rate of 7%, including 21% among children <2 years of age. Exposure to *H. pylori*–infected persons with gastroenteritis, particularly with vomiting, increased risk for new infection, and three quarters of definite or probable new infections were attributable to exposure to *H. pylori* infection with gastroenteritis. These findings indicate that in US immigrant homes, *H. pylori* transmission occurs in young children during household episodes of gastroenteritis.

Exposure to an infected household member with vomiting was associated with a 6-fold greater risk for new infection, whereas exposure to diarrhea elevated, but not significantly, the risk for new infection. These findings are consistent with prior research that shows that *H. pylori* is recovered reliably from vomitus (up to 30,000 CFU/mL) and can also can be grown from aerosolized vomitus collected at short distances (<1.2 m) (*13*). Epidemiologic investigations also implicate vomitus as an effective vehicle for gastro-oral transmission (*31*,*32*). Although found in diarrheal stools (*13*), *H. pylori* is not reliably grown from normal stools (*33*). The association between *H. pylori* and gastroenteritis is thus similar to that of other enteric pathogens that can be transmitted by vomitus or aerosolized vomitus or by the fecal-oral route (*34*,*35*). Although we cannot exclude other mechanisms of transmission in these homes, exposure to vomitus in an infected contact explained >50% of all new infections and >70% of definite and probable new infections.

Among our interests was identifying symptoms of new *H. pylori* infection. In experimental exposure, acute infection causes mild to moderate epigastric discomfort or dyspepsia in most study participants within 2 weeks, but symptoms are unlikely to be clinically detected (*36*). Although *H. pylori*–specific IgM antibodies may appear within 4 weeks, the frequency of this response is variable, particularly in children and when, as here, the time of infection is unknown (*37*). We did not observe a pronounced difference in the frequency or distribution of symptoms associated with new infection, although vomiting tended to be more frequent among persons with definite or probable new infections. Although the relatively small numbers of new cases may have limited the power of this analysis, no symptom complex was identified that would permit differentiation of acute *H. pylori* infection from other enteric processes. Because we did not establish the specific etiologic agent of gastroenteritis episodes, further studies are needed to more fully address this question.

Half of new infections were in children <2 years of age, and 2 of 3 were identified by a single unconfirmed stool conversion. Although *H. pylori* infection is acquired in early childhood, age of acquisition has been difficult to establish because of known limitations of existing noninvasive tests in very young children. In a Bogalusa, Louisiana, birth cohort, for example, the highest seroconversion rate (2%) was seen in children 4–5 years of age (*2*). Although stool antigen and urea breath tests are considered more accurate (*22*), studies in very young children are still limited. When the urea breath test was used, an annualized conversion rate more similar to ours (20%) was observed in the US–Mexican binational Pasitos cohort of children followed up from birth to 2 years (*38*). If, as suggested by this and other studies (*24*,*27*,*39*,*40*), acquisition with transient infection in early life often precedes persistent infection, rates of acquisition might be elevated when exposure to gastroenteritis is frequent.

Table 3. Risk factors for new infection in households with ≥1 *Helicobacter–pylori* infected participant*

Symptoms of *H. pylori*–infected household contact	Definite/probable new infections (n = 14 in 555 households)		All new infections (n = 23 in 566 households)	
	AOR	95% CI	AOR	95% CI
No GE	1.0		1.0	
GE w/ vomiting ± diarrhea	6.3	1.6–24.5	2.9	1.0–8.1
GE w/ diarrhea only	3.0	0.5–17.2	1.6	0.4–6.2

*AOR, adjusted odds ratio (random intercept model (household), adjusting for age, sleeping density, proportion of household completing both visits); CI, confidence interval; GE, gastroenteritis.

While the extent to which testing error confounds pediatric incidence studies is not clear, rates of acquisition among children in homes at high risk may nonetheless be meaningful measures of transmission risk.

Given the possibility of error in serologic and stool antigen tests, we cannot exclude the possibility that 30 new infections would occur by chance (*28*,*29*). Among those tested by serology, for example, the reversion rate was roughly equivalent to the seroconversion rate, which suggests that false-positive tests were occurring equally in the first and second screening. Because the predictive value of a single test in a low prevalence population may be <50%, we used a corroborative testing algorithm and restricted our risk factor analysis to those who had a confirmatory test of conversion. Despite the small number of cases, an association with exposure to *H. pylori* and gastroenteritis was highly significant. Although CIs were wide, the fact that risk estimates were uniformly strengthened in the subset of persons with corroborative test results lends validity to this approach, as well as underscoring the desirability of using a second test more routinely in incidence studies.

Although households that completed the study were largely representative of enrolled households, a substantial proportion of contacts in homes that completed the study either declined to participate (35%) or did not complete the second visit (15%). While rates of baseline *H. pylori* infection were virtually identical in those completing 1 or 2 visits, and participation rates were largely similar across household exposure profiles, we cannot exclude the possibility that the missing data were meaningful. If misclassification of gastroenteritis did occur, linkage with outcome is unlikely, since *H. pylori* infection status is not typically known. For these reasons, we assume that misclassification was random, minimizing the magnitude of true associations.

In summary, this study corroborates the conclusion that gastroenteritis, particularly with vomiting, in an *H. pylori*–infected person is a primary cause of transmission of *H. pylori* in humans. Despite some study limitations, the strength of association observed suggests an important milieu for future work to elucidate transmission pathways in low prevalence countries. As with other enteric infections such as hepatitis A, shigellosis, and cholera, *H. pylori* infection rates have decreased dramatically with improvements in sanitary infrastructure and household hygienic practices. Despite these trends, acquisition and infection are likely to remain prevalent in households with preexisting *H. pylori* infection, crowded living conditions, and frequent gastroenteritis.

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Serotype Competence and Penicillin Resistance in *Streptococcus pneumoniae*

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From 2003 to 2005, we prospectively collected 118 isolates of pneumococci belonging to 7 serotypes to investigate their competence under the influence of the synthetic competence-stimulating peptides. The degree of competence of the various serotypes differed significantly. Serotype 6B had the highest competence, followed by serotypes 14, 19F, 9V, 23F, 3, and 18C. Isolates belonging to serotype 6B had greater genetic diversity than isolates belonging to serotype 3, which has high genetic clustering. Isolates belonging to serotypes 3 and 18C that were 100% sensitive to penicillin were significantly less competent than isolates belonging to serotypes 6B, 14, 19F, 9V, and 23F, which were frequently resistant to penicillin. Under the 7-valent pneumococcal conjugate vaccine program, enhanced molecular surveillance of virulent clones with higher competence to detect serotype switching will become more important.

Streptococcus pneumoniae, a leading cause of bacteremia, sinusitis, otitis media, bacterial meningitis, and bacterial pneumonia, causes substantial illness and death in persons worldwide (*1*). In recent decades, the increase of *S. pneumoniae* strains resistant to β-lactam antimicrobial drugs and other classes of antimicrobial drugs has further complicated the treatment of pneumococcal infection (*2*). Although the current introduction of conjugate pneumococcal vaccine has successfully reduced invasive pneumococcal disease caused by the vaccine serotypes and effectively decreased the spread of antimicrobial drug–resistant isolates, pneumococcal infection remains a major issue, in light of the selective pressure that has been invoked by vaccination programs (*3*,*4*). At least 2 consequences have been noted since the large-scale use of 7-valent conjugate vaccine. First, serotypes not covered by the conjugate vaccine have increased both in nasopharyngeal colonization and clinical illness (serotype replacement) (*5*,*6*). Second, serotype switching can occur through recombination in naturally transformable clones and result in the acquisition of a nonvaccine capsule (*5*,*7*). Therefore, investigating how competent pneumococci can be for genetic transformation is useful because this factor plays a role in the evolution of *S. pneumoniae*, especially with respect to virulence and antimicrobial drug resistance (*8*,*9*). The ability of *S. pneumoniae* to undergo horizontal gene transfer leads to genetic diversity and helps the organism adapt to environmental changes. Since the discovery of competence-stimulating peptide (CSP) by Havarstein et al., the detailed mechanism of competence in *S. pneumoniae* has gradually been clarified (*10*). However, few studies have examined the different competence levels across a range of clinical isolates (*11*). Our aims in this study were to compare competence among clinical isolates of various serotypes and to increase our understanding of the role of competence in penicillin-resistant *S. pneumoniae*.

Materials and Methods

Bacterial Isolates

From January 2003 to December 2005, a total of 118 *S. pneumomiae* isolates were prospectively collected for this study. These isolates were recovered from various clinical

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specimens obtained from patients in 6 medical centers in Taiwan. The samples were collected from areas in which pneumococcal conjugate vaccine has not been widely implemented. The hospitals included National Taiwan University Hospital, Taipei (62 isolates); Taichung Veterans General Hospital, Taichung (19 isolates); People's Republic of China Medical College Hospital, Taichung (5 isolates); National Cheng-Kung University Hospital, Tainan (3 isolates); Chang-Gung Memorial Hospital, Kaohsiung (12 isolates); and Kaohsiung Veterans General Hospital, Kaohsiung (17 isolates). Among these isolates, 24 isolates (20.2%) were recovered from normally sterile body sites (21 isolates from blood, 2 isolates from pleural fluid, and 1isolate from peritoneal fluid); the rest were isolated from respiratory tract secretions.

Antimicrobial Drug Susceptibility Testing

The MICs of penicillin for all 118 *S. pneumoniae* isolates were determined by the agar dilution method and were interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) (*12*,*13*). Strains with an MIC ≤0.06 μg/mL were defined as susceptible, i.e., penicillin-susceptible *S. pneumoniae* (PSSP). Strains with an MIC of 0.12–1 μg/mL were defined as intermediately resistant, i.e., penicillin-intermediate *S. pneumoniae* (PISP), whereas those with an MIC ≥2 μg/mL were penicillin-resistant *S. pneumoniae* (PRSP).

Serotyping

The serotypes of isolates were determined by using the capsular swelling method (Quellung reaction). All antisera were obtained from the Statens Seruminstitut (Copenhagen, Denmark).

PFGE Analysis

Serotype 6B, which had the highest competence, and serotypes 3 and 18C, which had the lowest competence, were selected for pulsed-field gel electrophoresis (PFGE) analysis. PFGE was performed according to the method previously described (*14*). The DNA was digested with *Sma*I. Bands were stained with ethidium bromide and visualized with UV light. PFGE patterns that differed by ≤3 bands were defined as 1 PFGE type; isolates with the same PFGE patterns indicated indistinguishable strains, and those with 2 or 3 different bands indicated closely related strains (*15*).

Transformation of *S. pneumoniae*

All pneumococcal isolates were grown at 35°C in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY) in static culture in the presence of 5% CO_2. Bacteria were stored in THY and 10% glycerol. Transformations were performed as described previously with modification (*8*,*16*). Briefly, early log phase *S. pneumoniae* cultures in THY, pH 6.8 (pH adjusted with HCl), supplemented with 1 mmol/L $CaCl_2$ and 0.2% bovine serum albumin (BSA) were diluted 1:10 with THY, pH 8.0 (adjusted with NaOH), supplemented with 1 mmol/L $CaCl_2$ and 0.2% BSA. Synthetic 100 ng/mL CSP-1 or CSP-2, in 10 mmol/L glucose and 10% horse serum (Sigma, Saint Louis, MO, USA) were added followed by incubation for 15 min at 37°C. Plasmid pDL278, an *Escherichia coli/S. pneumoniae* shuttle vector that contained the pVA380–1 basic replicon and the pUC origin of replication (6,733 bp) was then added (1 μg/mL), and samples were incubated for 1 h at 35°C under 5% CO_2 before being spread on blood agar plates containing 500 μg/mL spectinomycin (*17*). Control experiments were carried out by using the same protocol without adding pDL278. The transformation frequencies were expressed as the $\log_{10}$ value of the percentage of transformed cells. Isolates with a $\log_{10}$ value of transformation frequencies less than −10 were defined as noncompetent isolates, and those larger than −4 were defined as high competent isolates. (Because the report of transformation frequency in *S. pneumoniae* is limited, we chose −4 as the cutoff point of high frequency based on the experience of transformation frequency in *Helicobacter pylori.* [*18*].)

Statistical Analysis

After log transformation, the data for competence (transformation frequency) exhibited a normal distribution ($p>0.05$). Thus, differences of competence between multiple serotypes and groups were tested by using 1-way analysis of variance with the Bonferroni method for post-hoc multiple comparisons. The Student *t* test was used when competence were compared between 2 groups. Linear regression was used to detect the trend of competence among the PSSP, PISP, and PRSP groups. The relationship of penicillin resistance with competence was analyzed in a logistic regression model which controlled for serotypes. χ^2 test or Fisher exact test was used for categorical variables to test significance between groups. Correlations between competence induced by CSP-1 and CSP-2 in each serotype were determined by Pearson's correlation coefficient. A p value < 0.05 was considered significant. All probabilities were 2-tailed. Data were reported as mean ± standard error of the mean (SEM) unless otherwise indicated.

Results

We found 7 serotypes among the 118 isolates: 6B (23 strains), 14 (20 strains), 23F (26 strains), 9V (14 strains), 19F (21 strains), 3 (11 strains), and 18C (3 strains). Of the

isolates, 26.3% were susceptible to penicillin; 54.2% were intermediately resistant and 19.5% were highly resistant.

Competence, Serotype, and Genetic Heterogeneity

Figure 1 shows the log-transformed means (±SEM) of the transformation frequencies of serotypes 6B, 14, 19F, 9V, 23F, 3, and 18C induced by CSP-1 and CSP-2. In general, competence induced by CSP-1 was higher than that induced by CSP-2, except for serotype 23F. Competence induced by CSP-1 was positively correlated with competence induced by CSP-2 (Pearson's correlation coefficient 0.84, $p<0.001$). Significant differences of competence were found among serotypes under induction by either CSP-1 or CSP-2 ($p<0.001$). Serotype 6B had the highest competence, followed by 14, 19F, 9V, 23F, 3, and 18C. Under the induction of CSP-1, competence in serotype 6B (-4.1 ± 0.2) was significantly higher than for serotypes 9V (-6.4 ± 0.4), 23F (-6.8 ± 0.5), 3 (-7.8 ± 0.7) and 18C (-8.4 ± 1.8); similarly, competence in serotype 14 (-5.4 ± 0.3) was significantly higher than for serotype 3 ($p<0.05$ by post-hoc analysis). Under the induction of CSP-2, competence in serotype 6B (-5.2 ± 0.3) was significantly higher than those in serotypes 23F (-6.7 ± 0.4), 3 (-8.1 ± 0.6), and 18C (-8.8 ± 1.6). Competence in serotypes 14 (-5.5 ± 0.3) and 19F (-6.2 ± 0.5) was significantly higher than for serotype 3, and competence in serotype 14 was significantly higher than for serotype 18C ($p<0.05$ by post-hoc analysis). The 23 isolates expressing serotype 6B displayed a high level of genetic diversity; this was illustrated by their division into 10 PFGE patterns (Figure 2). Among them, 1 main PFGE type accounted for 30.4% (7/23) of all isolates of serotype 6B. These 7 strains were closely related. In contrast, the 11 isolates expressing serotype 3 were genetically indistinguishable, which showed only 1 PFGE pattern. The result indicated that this serotype was highly clonal in Taiwan (Figure 2). The 3 isolates expressing serotype 18C had 2 PFGE types. Twenty-two isolates had a competence higher than 10^{-4} under the induction of CSP-1: 11 isolates of 6B, 4 isolates of 14, 4 isolates of 19F, and 3 isolates of 23F. Under the induction of CSP-1, isolates belonging to serotypes 6B, 14, 19F, and 23F were significantly associated with high competence when compared with isolates belonging to serotypes 9V, 3, and 18C (24.4%, 22/90 vs 0%, 0/28; $p = 0.002$). Six isolates had competence higher than 10^{-4} under the induction of CSP-2: 2 isolates of 6B, 3 isolates of 14, and 1 isolate of 23F. Under the induction of CSP-2, isolates belonging to serotypes 6B, 14, 19F, and 23F were not associated with high competence compared with isolates belonging to serotypes 9V, 3, and 18C (6.7%, 6/90 vs. 0%, 0/28; $p = 0.3$). Among all the 118 isolates, 111 (94.1%) became competent after the induction with CSP-1, and 112 (94.9%) became competent after the induction with CSP-2. Two isolates of 19F, 1 isolate of 23F, 1 isolate of serotype 18C, and 2 isolate of serotype 3 were noncompetent with either CSP-1 or CSP-2.

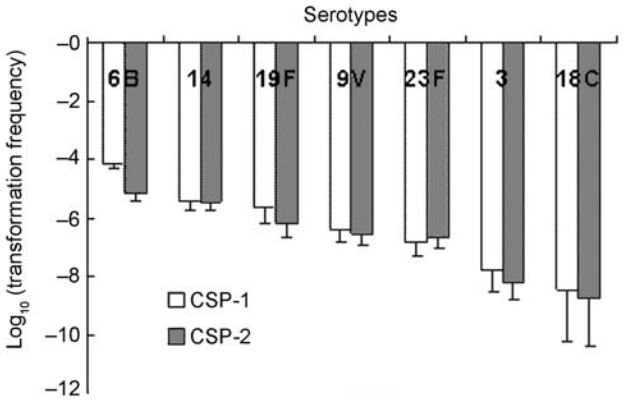

Figure 1. Competence (transformation frequency) induced by competence-stimulating peptide 1 (CSP-1) and CSP-2 in clinical isolates.

Competence and Penicillin Resistance

The distributions of PSSP, PISP, and PRSP in each serotype were significantly different ($p = 0.001$) (Table). The proportions of penicillin-susceptibility in serotypes 6B, 14, 19F, 9V and 23F were 8.7%, 35%, 19%, 21.4%, and 3.8%, respectively. (Table). While in serotypes 3 and 18C, the proportions of penicillin-susceptibility were 100%. Under the induction of either CSP-1 or CSP-2,

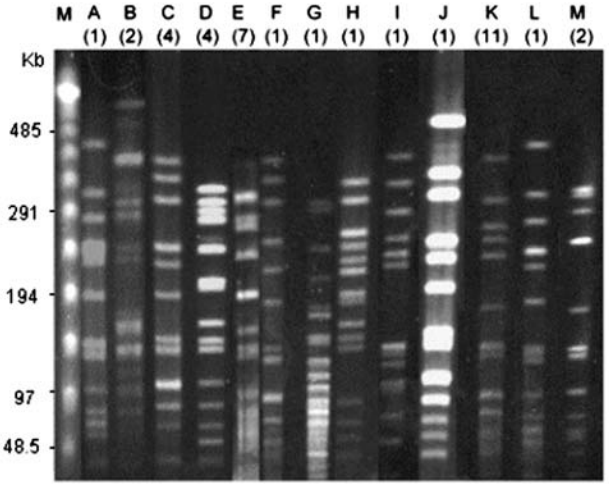

Figure 2. Pulsed-field gel electrophoresis (PFGE) types of 47 isolates of serotype 6B, serotype 3, and serotype 18C. Numbers in parentheses indicate the total number of isolates belonging to each PFGE type. Lane M, λ ladder; lanes A–J, 10 PFGE types found among 23 isolates of serotype 6B; lane K, 1 PFGE type found among 11 isolates of serotype 3; lanes L–M, 2 PFGE types found among 3 isolates of serotype 18C.

Table. Percentage of PSSP, PISP, and PRSP in each serotype*

	Serotype						
Degree of susceptibility	6B, n = 23 (%)	14, n = 20 (%)	19F, n = 21 (%)	9V, n = 14 (%)	23F, n = 26 (%)	3, n = 11 (%)	18C, n = 3 (%)
PSSP (MIC ≤0.06 μg/mL)	2 (8.7)	7 (35)	4 (19)	3 (21.4)	1 (3.8)	11 (100)	3 (100)
PISP (MIC 0.1–1μg/mL)	18 (78.3)	10 (50)	12 (57.1)	5 (35.7)	19 (73.1)	0	0
PRSP (MIC ≥2 μg/mL)	3 (13)	3 (15)	5 (23.8)	6 (42.9)	6 (23.1)	0	0

*PSSP, penicillin-susceptible *Streptococcus pneumoniae*; PISP, penicillin-intermediate *S. pneumoniae*; PRSP, penicillin-resistant *S. pneumoniae.*

isolates belonging to serotypes 3 and 18C that were not resistant against penicillin were significantly less competent than isolates belonging to serotypes 6B, 14, 19F, 9V, and 23F, which were frequently resistant to penicillin ($p<0.001$ for CSP-1, $p<0.001$ for CSP-2 by the Student *t* test) (Figure 3). Among all isolates belonging to serotype 6B, 14, 19F, 9V, and 23F, the proportions of PSSP, PISP, and PRSP were 16.3%, 61.5%, and 22.2%. Competence between PSSP, PISP, and PRSP was not significantly different ($p = 0.2$ for CSP-1, $p = 0.3$ for CSP-2) (Figure 4). By using the linear regression test to test for trend, competence was not significantly correlated with increasing penicillin resistance among isolates belonging to serotypes 6B, 14, 19F, 9V, and 23F ($p = 0.7$ for CSP-1, $p = 0.3$ for CSP-2). In a logistic regression model that controlled for serotypes, competence was not significantly correlated with penicillin resistance (odds ratio 0.8, $p = 0.3$, 95% confidence interval [CI] 0.58–1.17 for CSP-1; odds ratio 0.9, $p = 0.4$, 95% CI 0.58–1.25 for CSP-2).

Discussion

S. pneumoniae was the first pathogen to demonstrate the phenomenon of transformation (*19*). In 1944, Avery et al. proved that the genetic material in bacterial cells was DNA by using a transformation model in *S. pneumoniae* (*20*). Natural competence for genetic transformation in *S. pneumoniae* is mediated by a quorum sensing-regulated system. CSP, a heptadecapeptide pheromone, induces competence in growing cells at a critical cell density by activating the 2-component signal transduction system comDE (*10*). However, spontaneous competence has been observed only in some rough laboratory strains. Most clinical encapsulated isolates do not show competence unless synthetic CSP is added (*11*). In this study, we examined the levels of competence of various clinical isolates with the aid of synthetic CSP to gain insight into the association between competence and serotype and penicillin resistance.

Our results showed that different serotypes of *S. pneumoniae* possess different levels of competence. Serotype 6B was the most competent, consistent with our findings that these strains had high genetic diversity. On the other hand, serotypes 3 were less competent, consistent with our findings that these strains had low genetic heterogeneity. Large amounts of capsular polysaccharide have been reported to have an inhibitory effect on transformation in *S. pneumoniae* (*21*), and therefore relatively rich amounts of capsular polysaccharide in serotype 3 and 18C may block uptake of foreign DNA. Finding extremely low competence in serotype 3 could explain the limited genetic heterogeneity in serotype 3, which has also been observed in Canada, the United Kingdom, and the Netherlands (*22*). Serotype 3 was an infrequent pathogen among childhood pneumococcal diseases before the conjugate pneumococcal vaccination was implemented, even though the serotype is highly virulent (*22*,*23*); however, it is emerging as an important pathogen after the implementation of conjugate pneumococcal vaccination programs (*24*,*25*). We thought that pneumococcal conjugate vaccine could expand to include serotype 3 because of its high virulence and limited capacity to facilitate capsular transformation through horizontal DNA transfer of serotype 3 to better prevent severe childhood pneumococcal disease.

S. pneumoniae acquires mosaic penicillin-binding protein (PBP) genes from other *Streptococcus* species through a transformation to become penicillin-resistant (*26*). Isolates belonging to serotypes 3 and 18C are too low in competence to have a chance of acquiring the penicillin-resistance gene. Therefore, serotypes 3 and 18C are rarely resistant to penicillin (*27*). Among isolates belonging to 6B, 14, 19F, 9V, and 23F, no significant difference and association between competence and different level of penicillin resistance were found. Our explanations based on this finding are as follows: 1) A high level of competence is needed for acquiring the penicillin-resistance

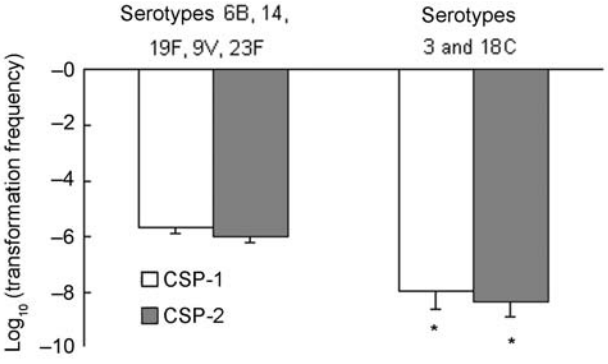

Figure 3. Competence (transformation frequency) induced by competence-stimulating peptide 1 (CSP-1) and CSP-2 between isolates belonging to serotypes 6B, 14, 19F, 9V, and 23F, and isolates belonging to serotypes 3 and 18C (*, $p<0.05$).

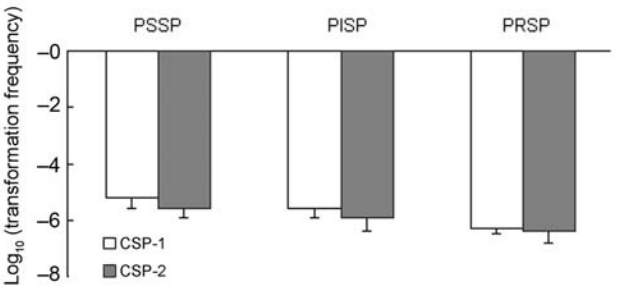

Figure 4. Competence (transformation frequency) induced by competence-stimulating peptide 1 (CSP-1) and CSP-2 among 3 groups: PSSP, PISP, and PRSP. PSSP, penicillin-susceptible *Streptococcus pneumoniae*; PISP, penicillin-intermediate *S. pneumoniae*; PRSP, penicillin-resistant *S. pneumoniae.*

gene. But, being more competent does not necessarily result in being penicillin resistant or in having a higher MIC of penicillin in isolates of serotypes 6B, 14, 19F, 9V, and 23F. 2) The development of penicillin resistance in *S. pneumoniae* is a multistep process. In addition to PBP, mutation or other non-PBP elements are also important, especially in the formation of PRSP (*28*,*29*). 3) After acquiring the penicillin-resistance gene, originally competent isolates might lose genetic components important for competence during high frequency of genetic transformation, resulting in isolates that are not competent.

Serotypes with higher competence are more likely to undergo recombinational exchanges to produce a new serotype or penicillin-resistant variant (*30*). The limitation of our study is examining competence for genetic transformation in *S. pneumoniae* by using in vitro assay. We are not sure if this method can reflect the real transformation capacity of *S. pneumoniae* in vivo. In our study, isolates belonging to serotypes 6B, 14, 19F, and 23F were associated with high competence, $>10^{-4}$, a finding that was in line with earlier studies that observed that serotype 6B, 14, 19F, and 23F frequently showed in vivo capsular transformation and related to be penicillin-resistant clinical isolates (*7*,*31*–*34*). The only exception is the relatively low competence of our 14 isolates belonging to serotype 9V, in which international Spain^{9V} had usually been reported to have in vivo capsular transformation (*35*,*36*). Certain serotypes that are frequently involved in capsular switching and penicillin resistance attributable to high competence should be further studied. To our knowledge, this is the first investigation into the relationship of competence and clinical characteristics in *S. pneumoniae*. After the introduction of the 7-valent pneumococcal conjugate vaccine, particular strains with genetic advantage may change their capsules from vaccine serotypes to nonvaccine serotype through capsular transformation (*5*,*7*). We suggest that enhanced surveillance of virulent clone with higher competence should allow the detection of serotype switching. This would be valuable for the long-term effectiveness of the conjugate vaccine.

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Staphylococcus aureus–associated Skin and Soft Tissue Infections in Ambulatory Care

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To describe the number and treatment of skin and soft tissue infections likely caused by *Staphylococcus aureus* in the United States, we analyzed data from the 1992–1994 and 2001–2003 National Ambulatory Medical Care Surveys and National Hospital Ambulatory Medical Care Surveys. Each year, data were reported by an average of 1,400 physicians, 230 outpatient departments, and 390 emergency departments for 30,000, 33,000, and 34,000 visits, respectively. During 2001–2003, the number of annual ambulatory care visits for skin and soft tissue infections was 11.6 million; the visit rate was 410.7 per 10,000 persons. During the study period, rates of overall and physician office visits did not differ; however, rates of visits to outpatient and emergency departments increased by 59% and 31%, respectively. This increase may reflect the emergence of community-acquired methicillin-resistant *S. aureus* infections.

Staphylococcus aureus is the almost-universal cause of furuncles, carbuncles, and skin abscesses and worldwide is the most commonly identified agent responsible for skin and soft tissue infections. *S. aureus* skin and soft tissue infections frequently begin as minor boils or abscesses and may progress to severe infections involving muscle or bone and may disseminate to the lungs or heart valves (i.e., endocarditis). Treatment of early infections consists of incising and draining the lesion, often accompanied by β-lactam antimicrobial drugs, which are also effective against β-hemolytic streptococci.

Strains resistant to β-lactam antimicrobial drugs, termed methicillin-resistant *S. aureus* (MRSA), were recognized from the 1960s through the 1990s as healthcare-associated (HA) pathogens (*1*). In the late 1990s, MRSA disease without established healthcare risk factors, called community-associated (CA)–MRSA, was increasingly reported in the literature (*2*,*3*). A study conducted in 2004 in emergency departments in 11 US cities found that MRSA was isolated from 59% of patients with skin and soft tissue infections (*4*). The biology of CA-MRSA appears to differ from that of HA-MRSA and CA–methicillin-susceptible *S. aureus* (MSSA), perhaps allowing CA-MRSA to cause disease other than that expected from MSSA (*5*–*8*). As HA-MRSA emerged, it likely did not merely replace HA-MSSA but led to an overall increase in *S. aureus* infections in healthcare settings (*9*–*11*).

Because most skin and soft tissue infections are treated in outpatient settings with empiric antimicrobial therapy, few studies have attempted to estimate the number of *S. aureus* skin and soft tissue infections, and none have evaluated the antimicrobial drugs prescribed for these conditions. Therefore, with regard to skin and soft tissue infections likely caused by *S. aureus*, we 1) estimated the number and rate of ambulatory care visits in the United States during 2 periods and examined any changes in these estimates between these periods; 2) described patient demographic characteristics; and 3) characterized antimicrobial and outpatient surgical therapy provided. Our results are based on a secondary data analysis of the 1992–1994 and 2001–2003 National Ambulatory Medical Care Surveys (NAMCS) and National Hospital Ambulatory Medical Care Surveys (NHAMCS).

Methods

Sample Design

NAMCS is a probability sample survey of office-based physicians in the United States, conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC). The US Bureau of the Census has been responsible for field operations and data collection since NAMCS became an annual survey in

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1989. Sample design, sampling variance, and estimation procedures of the NAMCS have been described (*12*). NHAMCS is an annual probability sample survey of hospital outpatient departments and emergency departments in the United States, first conducted in 1992 by CDC's National Center for Health Statistics. The US Census Bureau is responsible for field operations and data collection. The plan and operation of NHAMCS have been described (*13*).

Response Rates and Sample Size

From 1992 through 2003, response rates were 64%–73% for physician offices, 87%–91% for outpatient departments, and 90%–97% for emergency departments. The annual number of participating physicians was 1,000–1,800, outpatient departments 224–283, and emergency departments 364–425. The number of patient record forms completed each year by physician offices was 24,000–36,000, by outpatient departments 28,000–35,000, and by emergency departments 26,000–40,000. Estimates for skin and soft tissue infection visits are based on 3,374 sample records from 1992 through 1994 and 3,941 from 2001 through 2003.

Data Collection and Coding

The same patient record form is used for the physician office and outpatient department settings, whereas the emergency department form differs slightly. The patient record form contains patient demographic data and information about the visit, including cause of injury, diagnosis, ambulatory surgical procedures (NAMCS and NHAMCS outpatient department), medications, and disposition. As many as 3 diagnoses are coded according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) (*14*). During 2001–2003, 1–3 causes of injury were coded according to the Supplementary Classification of External Causes of Injury and Poisoning in the ICD-9-CM, and 1–2 ambulatory surgical procedures were coded to ICD-9-CM volume 3 (*14*). Cause of injury was not collected on the NAMCS and outpatient department patient record forms until 1995. From 1992 through 1994, 1–5 medications were recorded per visit; this number increased to 6 from 1995 through 2002 and 8 in 2003. Therapeutic classifications were based on the National Drug Code Directory (*15*,*16*). For this analysis, only 5 drugs per visit were included. A report describing the method and instruments used to collect and process drug information has been published (*17*).

Definitions

Skin and soft tissue infections likely caused by *S. aureus* are defined as any diagnoses assigned the ICD-9-CM codes shown in Table 1. These infections were selected because of their likelihood of being caused by *S. aureus* as determined by the authors and as they appeared in a medical textbook (*18*). The ICD-9-CM code for *Staphylococcus*, 41.1, was not included because it is used as an additional code to identify the bacterial agent in diseases classified elsewhere. Few records with this code were found in NAMCS and NHAMCS data, most likely because cultures were either not performed or the results were not available at the time of the visit.

The 1992–1994 denominators used in calculating the visit rates for age, sex, race, and geographic region are based on the Census Bureau estimates of the civilian, noninstitutional population of the United States as of July 1, 1992; July 1, 1993; and July 1, 1994, respectively. The 2001–2003 denominators are based on post–Census 2000 estimates of the civilian noninstitutional population of the United States. Population estimates of metropolitan statistical area status are based on data from the 2001, 2002, and 2003 National Health Interview Surveys, National Center for Health Statistics, adjusted to the US Census Bureau definition of core-based statistical areas. Denominators used to compute estimates of visit rates by expected source of payment were obtained from the 2001, 2002, and 2003 National Health Interview Surveys. Persons who reported multiple insurance categories were counted in each category reported, with the exception of Medicaid and the State Children's Health Insurance Program, which were combined into a single category. Denominator data for type of insurance were not available for 1992 through 1994.

In the emergency department, "wound care" can be checked on the patient record form and includes cleaning, debridement, and dressing of burns; repair of lacerations with skin tape or sutures; removal of foreign bodies; excisions; and incision and drainage of wounds provided at the visit. Physician office and outpatient department forms have space to write in ambulatory surgical procedures. ICD-9-CM procedure codes were combined to describe the surgical management of skin and soft tissue infections.

Statistical Analyses

NAMCS and NHAMCS data were weighted to produce national estimates, and data were combined in 2 groups of 3 years each (1992–1994 and 2001–2003) to provide more reliable estimates. The NAMCS weight includes 4 components: selection probability, nonresponse adjustment, physician-population weighting ratio adjustment, and weight smoothing. Starting with 2001 data, the adjustment for NAMCS physicians who did not provide patient record forms differs from the adjustment used in prior years by taking into account additional characteristics of the physician's practice. Previously, these characteristics were assumed to be the same for physicians who provided information about patient visits and those who did not. The

Table 1. Average annual percentage and rate of ambulatory care visits for selected skin and soft tissue infections, by diagnosis, United States*

Diagnosis	ICD-9-CM code	1992–1994 % visits†	1992–1994 95% CI	1992–1994 No. visits/ 10,000 persons/y	1992–1994 95% CI	2001–2003 % visits†	2001–2003 95% CI	2001–2003 No. visits/ 10,000 persons/y	2001–2003 95% CI
All visits		NA	NA	376.3	340.4–412.3	NA	NA	410.7	368.7–452.7
Inflammatory disease of breast	611.0	3.9	2.6–5.3	14.8	9.6–20.0	3.2	2.1–4.9	13.2	7.4–19.0
Carbuncle and furuncle	680	3.0	1.7–4.2	11.1	6.2–16.0	3.8	2.8–5.1	15.6	10.8–20.4
Cellulitis and abscess of finger and toe	681	9.4	7.7–11.2	35.5	28.0–43.1	9.7	7.6–12.3	39.8	29.6–50.1
Other cellulitis and abscess	682	46.2	42.6–49.9	174.0	150.4–197.7‡	53.2	49.1–57.2	218.4	188.7–248.1‡
Impetigo	684	14.3	11.5–17.2	53.9	42.2–65.6‡	8.9	6.9–11.6	36.7	26.4–47.0‡
Unspecified local infection of skin and subcutaneous tissue	686.9	9.6	7.2–11.9	36.1	26.9–45.3	8.0	6.3–10.1	32.7	24.8–40.6
Other specified diseases of hair and hair follicles	704.8	9.5	7.6–11.4	35.7	28.2–43.2	10.9	8.7–13.7	44.9	33.9–55.9
Hydradenitis	705.83	1.8	0.8–2.7	6.6	2.9–10.3	2.0	1.3–3.3	8.3	4.2–12.4
Other skin and soft tissue infections	§	3.4	1.7–5.1	12.9	6.6–19.2	1.9	1.1–3.2	7.8	3.5–12.1

*ICD-9-CM, International Classification of Diseases, Ninth Revision, Clinical Modification; CI, confidence interval; NA, not applicable.
†Total exceeds 100% because >1 diagnosis may be reported per visit.
‡1992–1994 95% CI overlaps with 2001–03 95% CI, but p<0.05.
§Includes ICD-9-CM codes 683 (acute lymphadenitis), 686.0 (pyoderma), 728.0 (myositis), 771.4 (omphalitis of the newborn), and 771.5 (neonatal infective mastitis).

NHAMCS weight includes 3 components: selection probability, nonresponse adjustment, and ratio adjustment to fixed totals. SUDAAN statistical software was used for all statistical analyses (*19*).

The determination of statistical significance was based on the 2-tailed *t* test (0.05 level of significance). The Bonferroni inequality was used to establish the critical value for statistically significant differences based on the number of possible comparisons within a particular variable (or combination of variables) of interest. Terms relating to differences such as "greater than" or "less than" indicate that the difference is statistically significant. The standard errors used to calculate the 95% confidence intervals (CIs) around the estimates took into account the complex sample designs of the NAMCS and NHAMCS. Estimates based on <30 cases in the sample data did not meet standard of reliability or precision and are indicated in the tables (*20*).

To determine which factors were independently associated with a diagnosis of skin and soft tissue infection, a logistic regression analysis that included all visits was performed. The dependent variable was defined as a diagnosis of skin and soft tissue infection. The model contained the following independent variables: setting type, age, sex, race, expected source of payment, and geographic region.

Before 2003, NAMCS and NHAMCS were exempt from Institutional Review Board review. In February 2003, NAMCS and NHAMCS protocols were approved by CDC's National Center for Health Statistics Research Ethics Review Board. Waivers were granted for the requirements to obtain informed consent of patients and patient authorization for release of patient medical record data by healthcare providers.

Results

During 2001–2003, a total of 11.6 million annual visits were made to US ambulatory care providers for selected skin and soft tissue infections, representing 1.0% (95% CI 0.9–1.1) of all visits; the visit rate was 410.7 per 10,000 persons. A comparison of the 1992–1994 and 2001–2003 visit rates showed no difference in the rates of overall and physician office visits during the study period; however, the rates for outpatient and emergency department visits increased by 59% and 31%, respectively (Figure). Tables 2 and 3 show the visit rates and percentage distributions for skin and soft tissue infections according to characteristics of patients, providers, and visits. The proportion of visits made to physician offices decreased from 1992–1994 through 2001–2003, while the proportion of visits to emergency and outpatient departments increased. More than half of all visits (56.2%, 95% CI 52.2–60.2) were initial visits, 33.3% (95% CI 29.6–37.3) were follow-up visits, and 10.5% (95% CI 7.9–13.7) were of unknown episode. No differences in visit rates were found according to sex,

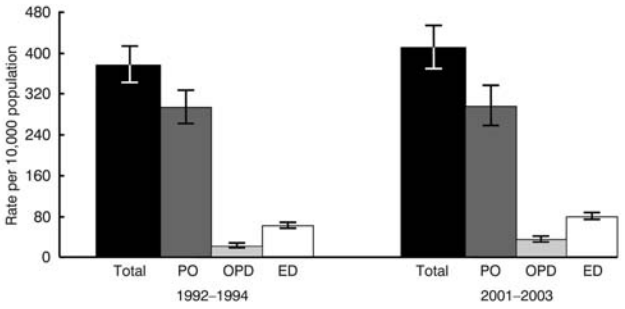

Figure. Average rates for annual ambulatory care visits for skin and soft tissue infections, by setting, United States, 1992–1994 and 2001–2003. p<0.001 for rates for outpatient and emergency department visits. PO, physician office; OPD, outpatient department; ED, emergency department. Error bars indicate 95% confidence intervals.

race, or metropolitan statistical area status. Rates were higher for children and adolescents 2–18 years of age than for persons ≥65 years of age and were higher for those residing in the South than those in the Midwest. A greater proportion of visits were made by female patients and a higher proportion occurred in the South than in the other 3 regions. Private insurance was the most frequently recorded expected source of payment, accounting for half of the visits. The visit rates for Medicare and Medicaid patients were higher than for those with private or no insurance. The proportions of visits made by patients eligible for Medicaid (23.9%, 95% CI 19.5–29.0) and patients with no insurance (15.4%, 95% CI 11.8–19.9) were higher for outpatient departments (14.5%, 95% CI 10.7–19.2) than physician offices (4.6%, 95% CI 2.8–7.2). When rates for 1992–1994 were compared with rates for 2001–2003, no differences were observed overall or by age, sex, or race; however, rates increased in metropolitan statistical areas and in the South and decreased in the Midwest.

Skin and soft tissue infection visits by diagnosis are displayed in Table 1. During 2001–2003, "other cellulitis and abscess" was diagnosed at 53.2% of visits; the visit rate for this diagnosis had increased by 26% since 1992–1994. In contrast, the visit rate for impetigo decreased by 32% during the study period.

Approximately 22.0% (95% CI 19.2–25.0) of visits for skin and soft tissue infection were related to injury. However, the cause of injury is not linked to diagnosis on the patient record form. During 2001–2003, the leading causes of injury were natural and environmental factors (including insect and animal bites) (22.4%, 95% CI 16.7–29.4), being unintentionally cut or pierced by instruments or objects (10.2%, 95% CI 6.7–15.1), and being accidentally struck against or struck by objects or persons (8.4%, 95% CI 5.1–13.5).

In the emergency department, wound care was provided at 41.6% (95% CI 36.7–46.7) of visits for injury-related skin and soft tissue infection. For injury- and illness-related skin and soft tissue infection visits, wound care was provided at 31.3% (95% CI 28.6–34.2) of visits. In physician office and outpatient department settings, procedures related to the surgical management of skin and soft tissue infections were ordered, scheduled, or performed at 9.6% (95% CI 7.3–12.4) of visits.

Antimicrobial drugs were prescribed at 64.6% (95% CI 60.8–68.2) of visits for skin and soft tissue infections during 2001–2003. Between 1992–1994 and 2001–2003, no differences were found in antimicrobial drug prescribing rates overall or for selected therapeutic subclasses, except for cephalosporins, which were prescribed at a higher rate during 2001–2003 and lincosamides/macrolides, which were prescribed at a higher rate during 1992–1994 (Table 4).

During 2001–2003, of all visits for skin and soft tissue infections, 4.0% (95% CI 3.0–5.3) resulted in hospital admission; when limited to visits to the emergency department only, the percentage was 13.6% (95% CI 10.5–14.7). These percentages did not differ from those observed during 1992–1994, which were 3.6% (95% CI 2.6–4.7) and 12.6% (95% CI 2.5–14.7), respectively. For all visits for skin and soft tissue infection, no follow-up was planned for 7.4% (95% CI 5.6–9.6), and referral to another physician was made for 14.4% (95% CI 12.4–16.6).

A multivariate model of factors associated with a skin and soft tissue infection diagnosis for 2001–2003 showed independent associations for the following: emergency department setting, male sex, payment by Medicaid, and residence in the South and West (Table 5). The results from the overall model were significant (p<0.001).

Discussion

We estimate 11.6 million ambulatory healthcare visits for skin and soft tissue infections possibly due to *S. aureus* in the United States each year from 2001 through 2003. No change in the overall visit rate for skin and soft tissue infections was found when compared with 1992–1994; however, an increase in these visit rates was observed in hospital emergency and outpatient departments, many for infections coded as cellulitis or abscess. This trend is consistent with findings from a study conducted in a Los Angeles emergency department, where the prevalence of MRSA among patients with skin and soft tissue infections rose from 29% in 2001–2002 to 64% in 2003–2004 (*21*). These data indicate that the number of *S. aureus* skin and soft tissue infections is substantial and that the emergence of CA-MRSA may affect ambulatory healthcare in the United States. Although we did not identify the causes of the infections, the increase in the rate of visits for cellulitis or abscesses

Table 2. Average annual number, percent distribution, and rate of ambulatory care visits for selected skin and soft tissue infections, by selected patient, provider, and visit characteristics, United States, 1992–1994*

Characteristic	Visits, in thousands (n)	% Distribution	95% CI	Visits/10,000 persons/y†	95% CI
All visits	9,601	100.0	NA	376.3	340.4–412.3
Setting					
Physician office	7,481	77.9	75.5–80.4	293.2	260.0–326.4
Outpatient department	558	5.8	4.7–7.0	21.9	17.2–26.5
Emergency department	1,562	16.3	14.4–8.2	61.2	55.0–67.5
Patient age, y					
<2	455	4.7	3.4–6.1	569.0	404.8–733.1
2–18	2,050	21.4	18.3–24.4	323.2	265.0–381.4
19–44	3,514	36.6	33.4–39.8	340.8	297.7–384.0
45–64	1,842	19.2	16.5–21.9	371.6	309.9–433.3
≥65	1,741	18.1	15.5–20.8	561.0	461.8–660.2
Patient sex					
Female	5,351	55.7	52.0–59.5	408.5	361.2–455.8
Male	4,250	44.3	40.5–48.0	342.4	297.9–386.8
Patient race					
White	8,186	85.3	82.1–88.5	386.6	345.8–427.4
Black or African American	1,158	12.1	9.8–14.3	360.0	289.6–430.3
Other	256‡	2.7‡	0.6–4.7	229.4‡	49.7–409.1
Provider region					
Northeast	2,026	21.1	17.2–25.0	403.4	320.1–486.7
Midwest	2,697	28.1	23.7–32.5	433.3	352.0–514.5
South	2,810	29.3	25.0–33.6	326.9	270.2–383.7§
West	2,068	21.5	17.7–25.4	364.8	291.9–437.7
Provider statistical area					
Metropolitan	7,148	74.5	67.9–81.0	357.9	322.8–393.0§
Nonmetropolitan	2,454	25.6	19.0–32.1	442.8	306.2–579.5
Payment					
Private insurance	4,601	47.9	44.1–51.8	¶	NA
Medicare	1,413	14.7	12.4–17.0	¶	NA
Medicaid or SCHIP	1,409	14.7	12.5–17.0	¶	NA
Uninsured#	1,575	16.4	13.3–19.5	¶	NA
Other**	603	6.3	4.8–7.8	¶	NA

*Numbers may not add to totals due to rounding. CI, confidence interval; NA, not applicable; SCHIP, State Children's Health Insurance Program.
†Visit rates for age, sex, race, and region are based on the average of US Census Bureau estimates of the civilian noninstitutional population of the United States, for July 1, 1992, July 1, 1993, and July 1, 1994.
‡Does not meet standard of reliability or precision.
§95% CI for 1992–1994 and 2001–2003 overlap, but $p<0.05$.
¶Denominator data not available.
#Includes self-pay, charity, and no charge.
**Includes Worker's Compensation, other payment, unknown, and blank.

may in part reflect the emergence of CA-MRSA. Many reports of CA-MRSA skin and soft tissue infections have been documented in either closed populations with frequent skin-to-skin contact (*22–25*) or emergency department patients and patients admitted through an emergency department (*4,8,25*). Published reports have indicated that CA-MRSA strains, especially those with the Panton-Valentine leukocidin toxin, are more likely to cause abscesses with a necrotic center that progress rapidly (*6–8*). This rapid progression of lesions, frequently described as spider bites (*26,27*), may lead persons to seek care in emergency departments rather than physician offices. A possible explanation for the increased visits to outpatient departments but not physician offices is differences in certain patient demographic and medical characteristics (*28,29*).

For all settings during 2001–2003, skin and soft tissue infections were independently associated with Medicaid reimbursement relative to private insurance. For all visits made to emergency and outpatient departments in 2003, utilization rates were about 4 times higher for Medicaid recipients than for those with private insurance (*28,30*); however, no difference was found for those who visited physician offices (*29*). CA-MRSA might disproportionately affect particular socioeconomic groups who are more likely to seek care in certain settings, which in turn might increase skin and soft tissue infection visit rates to some ambulatory care settings (*5*). However, this finding does not mean that visit rates to other ambulatory care settings will not increase as CA-MRSA continues to emerge.

According to the results of our multivariate analysis, the demographic groups at greatest risk for skin and soft tissue infections likely caused by *S. aureus* are patients who are male, reside in the South or West, and receive Medicaid. In contrast to the age groups affected by most health conditions, the oldest age groups are at lower risk. Because of the contagious nature of *S. aureus* strains responsible for skin and soft tissue infection, younger men who have skin-to-skin contact, such as those who play on athletic teams, may be more likely to acquire the infection (*23*,*24*). The association with certain geographic regions may reflect the distribution of demographic groups at highest risk or, alternatively, climate factors (e.g., higher heat and humidity) conducive to skin and soft tissue infections.

As CA-MRSA continues to emerge, monitoring its effect on therapy and whether clinicians are responding appropriately will be helpful. For abscesses, incision and drainage constitute the most important form of primary therapy (*31*,*32*). For >30% of all visits to emergency departments and 10% of visits to outpatient departments and physician offices, wound care, which could include incision and drainage, was provided. Logically, provision of wound care would be higher in emergency departments than in physician offices or outpatient departments, given that patients with more severe infections are generally referred to this setting. However, another explanation of the difference in the 3 settings may be manner of data collection. In the emergency department, wound care is indi-

Table 3. Average annual number, percent distribution, and rate of ambulatory care visits for selected skin and soft tissue infections, by selected patient, provider, and visit characteristics, United States, 2001–2003*

Characteristic	Visits, n in thousands	% Distribution	95% CI	Visits/10,000 persons/y†	95% CI
All visits	11,618	100.0	NA	410.7	368.7–452.7
Setting					
Physician office	8,370	72.0	68.8–75.1	295.9	255.9–335.9
Outpatient department	986	8.5	7.1–10.2	34.9	29.1–40.6
Emergency department	2,262	19.5	17.3–21.9	80.0	72.3–87.7
Patient age, y					
<2	576	5.0	3.4–7.3	721.5	434.2–1008.9
2–18	2,292	19.7	16.8–23.1	333.7	272.6–394.7
19–44	3,921	33.7	30.2–37.5	369.6	315.8–423.5
45–64	2,793	24.0	20.4–28.1	422.2	346.7–497.6
≥65	2,036	17.5	13.7–22.1	599.2	431.9–766.6
Patient sex					
Female	6,403	55.1	51.5–58.7	442.0	388.2–495.7
Male	5,216	44.9	41.3–48.5	377.9	329.1–426.7
Patient race					
White	9,427	81.1	77.2–84.6	412.1	365.6–458.5
Black or African American	1,635	14.1	11.5–17.1	462.8	362.3–563.3
Other	556	4.8	3.0–7.7	296.1	149.3–442.8
Provider region					
Northeast	2,323	20.0	16.1–24.5	435.3	333.9–536.8
Midwest	2,046	17.6	14.5–21.2	319.3	255.7–382.8
South	4,641	40.0	34.9–45.3	459.7	377.3–542.2‡
West	2,608	22.5	18.7–26.7	404.4	325.0–483.9
Provider statistical area					
Metropolitan	9,775	84.1	79.3–88.0	425.4	374.9–476.0‡
Nonmetropolitan	1,843	15.9	12.0–20.7	347.1	249. 1–445.1
Payment					
Private insurance	5,853	50.4	46.0–54.8	302.8	264.0–341.5
Medicare	2,049	17.6	13.8–22.2	591.2	425.6–756.7
Medicaid or SCHIP	1,889	16.3	13.4–19.7	675.3	530.9–819.7
Uninsured§	990	8.5	6.9–10.4	242.9	192.9–293.0
Other¶	837	7.2	5.6–9.3	#	NA

*Numbers may not add to totals due to rounding. CI, confidence interval; NA, not applicable; SCHIP, State Children's Health Insurance Program.
†Visit rates for age, sex, race, and region are based on the average of US Census Bureau estimates of the civilian noninstitutional population of the United States, for July 1, 2001, July 1, 2002, and July 1, 2003; population estimates of metropolitan statistical area status are based on data from the 2001, 2002, and 2003 National Health Interview Survey, National Center for Health Statistics, adjusted to the US Census Bureau definition of core-based statistical areas; and denominator data for expected source of payment are based on the 2001, 2002, and 2003 National Health Interview Survey, National Center for Health Statistics.
‡95% CI for 1992–1994 and 2001–2003 overlap, but p<0.05.
§Includes self-pay, charity, and no charge.
¶Includes Worker's Compensation, other payment, unknown, and blank.
#Denominator data not available.

Table 4. Average annual antimicrobial prescribing rates at ambulatory care visits for selected skin and soft tissue infections, by therapeutic subclass, United States*

	1992–1994		2001–2003	
Therapeutic subclass†	No. prescriptions/ 10,000 visits/y	95% CI	No. prescriptions/ 10,000 visits/y	95% CI
All visits	6,899.9	6431.5–7368.3	7,298.6	6870.7–7726.5
Cephalosporins	3,039.3	2,704.5–3374.1‡	3,558.3	3,191.2–3,925.4‡
Penicillins	1,098.7	826.1–1371.3	1,404.2	1,141.0–1,667.4
Lincosamides and macrolides	1,377.7	1,081.9–1673.5	668.7	508.6–828.8
Quinolones	§	NA	646.3	472.8–819.8
Sulfonamides and related compounds, antibacterial agents, miscellaneous	580.8	390.9–770.7	542.2	361.9–722.5
Tetracyclines	134.2	73.6–194.8	258.3	146.8–369.8

*CI, confidence interval; NA, not applicable.
†Therapeutic subclass is based on the standard 4-digit drug classification used in the National Drug Code Directory, 1985 and 1995 editions, respectively.
‡1992–1994 95% CI overlaps with 2001–2003 95% CI, but p<0.05.
§Does not meet standard of reliability or precision.

cated by checking a box, whereas in the other settings the procedure is written in. Write-in items generally have a higher nonresponse rate than check-box items (*33*).

With the continued emergence of CA-MRSA, the clinical management of skin and soft tissue infections has now returned to the basic principles of surgical drainage and debulking, wound culture, and the use of older antimicrobial agents other than β-lactams (*34*). However, our results indicate that β-lactam drugs consisting of cephalosporins and penicillins remain the most commonly prescribed therapy for skin and soft tissue infections and that the rate of use of cephalosporins increased over the 12-year study period. A recent study found that for 57% of patients seen in emergency departments for skin and soft tissue infections associated with MRSA, the infecting isolate was resistant to the agent prescribed (*4*). Before the emergence of CA-MRSA, the most appropriate form of antimicrobial therapy for skin and soft tissue infection was β-lactams (assuming penicillins consisted of antistaphylococcal agents). Now clinicians must take into account local and regional rates of CA-MRSA and consider the use of agents such as clindamycin (a lincosamide) or trimethoprim-sulfamethoxazole in the empiric treatment of skin and soft tissue infections (*32*). Periodic monitoring of antimicrobial drug use may be helpful as CA-MRSA continues to emerge.

Our study has several limitations. Most important is the fact that these ICD-9-CM codes have not been validated as a method for tracking skin and soft tissue infections likely to be caused by *S. aureus*, much less infections caused by CA-MRSA. In addition, whether the baseline risks for skin and soft tissue infections were similar between the 2 periods studied is unknown. Rates for conditions such as diabetes, peripheral vascular disease, traumatic injuries, and homelessness might differ for the 2 periods, which would obscure any actual increase in skin infections due to MRSA. Although 3 years of data were combined, some estimates were not presented because they were unreliable, and some estimates for diagnoses, drugs, and procedures were aggregated into broader categories to attain reliability. Because the design of NAMCS and NHAMCS does not allow for patient follow-up, some cases may have been counted multiple times. Because diagnosis cannot be associated with a particular drug, we could only assume that the antimicrobial drug listed was prescribed for the skin or soft tissue infection diagnosis recorded at the same visit. Procedure data are collected differently in emergency departments than in physician offices and hospital outpatient departments and, therefore, are not comparable. Skin and soft tissue infections misdiagnosed as spider bites

Table 5. Factors associated with ambulatory care visits for skin and soft tissue infection

Factor	Adjusted odds ratio (95% confidence interval)
Setting	
Physician office	Referent
Emergency department*	2.0 (1.8–2.4)
Outpatient department	1.2 (1.0–1.4)
Age, y	
<2	0.9 (0.6–1.4)
2–18	1.2 (1.0–1.6)
19–44	1.2 (1.0–1.5)
45–64	Referent
≥65	0.7 (0.5–1.0)
Sex	
Female	Referent
Male*	1.2 (1.0–1.4)
Payment	
Private insurance	Referent
Medicaid*	1.4 (1.1–1.8)
Medicare	1.3 (0.9–1.9)
Uninsured	1.2 (0.9–1.5)
Other insurance	1.0 (0.7–1.3)
Region	
Midwest	Referent
Northeast	1.1 (0.9–1.5)
South*	1.3 (1.1–1.7)
West*	1.4 (1.1–1.8)

*p<0.05.

may not have been captured in NAMCS or NHAMCS. We found that the rate for all visits assigned a cause-of-injury E-code of 905.1 (venomous spiders) increased significantly, from 2.7 per 10,000 persons (95% CI 1.5–3.9) during 1992–1994 to 8.4 (95% CI 4.9–11.9) during 2001–2003.

In conclusion, we found that the number of skin and soft tissue infections is increasing in hospital emergency and outpatient departments; this increase may reflect the emergence of CA-MRSA. However, despite these increases, changes in the therapeutic approach to these infections are not apparent. These findings may serve as a baseline for future analyses to track the continued emergence and effect of CA-MRSA on ambulatory healthcare and to monitor how clinicians adapt and treat these patients.

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Humans as Reservoir for Enterotoxin Gene–carrying *Clostridium perfringens* Type A

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We found a prevalence of 18% for enterotoxin gene–carrying (*cpe*+) *Clostridium perfringens* in the feces of healthy food handlers by PCR and isolated the organism from 11 of 23 PCR-positive persons by using hydrophobic grid membrane filter-colony hybridization. Several different *cpe* genotypes were recovered. The prevalence was 3.7% for plasmidial IS*1151-cpe*, 2.9% for plasmidial IS*1470*-like-*cpe*, 0.7% for chromosomal IS*1470-cpe*, and 1.5% for unknown *cpe* genotype. Lateral spread of *cpe* between *C. perfringens* strains was evident because strains from the same person carried IS*1470*-like *cpe* but shared no genetic relatedness according to pulsed-field gel electrophoresis analysis. Our findings suggest that healthy humans serve as a rich reservoir for *cpe*+ *C. perfringens* type A and may play a role in the etiology of gastrointestinal diseases caused by this organism. The results also indicate that humans should be considered a risk factor for spread of *C. perfringens* type A food poisoning and that they are a possible source of contamination for *C. perfringens* type A food poisoning.

Clostridium perfringens is classified into 5 types (A–E) on the basis of its ability to produce ≥1 of the major lethal toxins α, β, ε, and ι (*1*). Enterotoxin (CPE)-producing (*cpe*+) *C. perfringens* type A is reported continuously as 1 of the most common food poisoning agents worldwide (*2*–*4*). An increasing number of reports also implicate the organism in 5%–15% of antimicrobial drug–associated diarrhea (AAD) and sporadic diarrhea (SD) cases in humans as well as diarrhea cases in animals (*1*,*5*–*9*). Most food poisoning strains studied to date carry *cpe* in their chromosomes; isolates from AAD and SD cases bear *cpe* in a plasmid (*10*,*11*). Furthermore, genetic studies have shown that in *C. perfringens* strains with the chromosomally located *cpe*, IS*1470* sequences are found upstream and downstream of *cpe* (*12*,*13*). However, in strains with *cpe* in the plasmid, 2 different genetic arrangements (either IS*1151* or IS*1470*-like sequences) have been recognized downstream of *cpe* (*11*,*14*).

Why *C. perfringens* strains with *cpe* located on chromosomes or plasmids cause different diseases has not been satisfactorily explained. However, the relatively greater heat resistance of the strains with chromosomally located *cpe* is a plausible explanation for these strains' survival in cooked food, thus causing instances of food poisonings (*15*). The presence of *C. perfringens* strains with chromosomally located *cpe* in 1.4% of American retail food indicates that these strains have an access to the food chain (*16*). The sources and routes of contamination are unclear.

An explanation for the strong association between *C. perfringens* strains with plasmidially located *cpe* and cases of AAD and SD disease may be in vivo transfer of the *cpe* plasmid to *C. perfringens* strains of the normal intestinal microbiota (*17*). Thus, a small amount of ingested *cpe*+ *C. perfringens* would act as an infectious agent and transfer the *cpe* plasmid to *cpe*– *C. perfringens* strains of the normal microbiota. This process would result in the persistence of *cpe*+ *C. perfringens* in the intestines. Chronic exposure to CPE would explain the severity and long duration of symptoms (*17*). Conjugative transfer of the *cpe* plasmid has been demonstrated in vitro (*18*), but currently no data exist on lateral transfer of *cpe* in vivo, and whether *cpe*+ strains that cause AAD and SD are resident in the gastrointestinal tract or acquired before onset of the disease is unknown.

Although the ubiquitous distribution of *C. perfringens* in nature is well documented, the epidemiology of *cpe*+ strains has not yet been established. Less than 5% of global

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C. perfringens isolates are estimated to carry *cpe* (*1*), and the prevalence of different *cpe* genotypes in specific ecologic niches is not known. In this study, healthy persons were screened for fecal carriage of *cpe*, and *cpe*+ strains were further isolated by using hydrophobic grid membrane filter-colony hybridization. The *cpe* genotype and location of *cpe* were determined from strains by detecting different insertion sequence (IS) elements attached to *cpe* (plasmid types IS*1151*-*cpe* and IS*1470*-like-*cpe* and chromosomal type IS*1470*-*cpe*). The genetic relationship between *cpe*+ and *cpe*– *C. perfringens* isolates obtained from *cpe* carriers was assessed with pulsed-field gel electrophoresis (PFGE).

Materials and Methods

Fecal Samples

A total of 136 fecal samples, 102 (75%) from female food handlers and 34 (25%) from male food handlers, were collected from food handlers in southern Finland during summer 2003. These persons reported no gastrointestinal symptoms at the time of sampling. Their ages ranged from 15 to 65 years (mean 30 years, median 24 years). The samples were kept at –70°C until investigated.

Detection and Isolation

Each fecal sample (1 g) was divided into 2 tubes that contained freshly prepared thioglycollate (40 mL) (Oxoid, Basingstoke, UK). One tube was heated at 75°C for 20 min; the other was left unheated. After anaerobic incubation at 37°C for 20–22 h, the presence of *cpe* in each tube was determined by nested PCR (*19*). Persons with a *cpe*-positive fecal sample are hereafter referred to as *cpe* carriers.

Hydrophobic grid membrane filter-colony hybridization (HGMF-CH) (*20*) was used to isolate *cpe*+ *C. perfringens* from *cpe* carriers. In addition to probe-positive colonies, those showing no hybridization signals but having a typical color for *C. perfringens* colonies (usually black, occasionally gray or grayish yellow) were isolated from each sample to obtain a collection of both *cpe*+ and *cpe*– *C. perfringens* from a single sample and to further study the genetic relatedness of these isolates by PFGE (see below).

The isolates were subjected to PCR to determine the toxinotype (A–E) and the presence of *cpe* (*21*). *C. perfringens* strains NCTC 8239, ATCC 3626, CCUG 2036, CCUG 2037, and CCUG 44727 were used as positive controls.

Molecular Typing

C. perfringens isolates that possessed *cpe* were further studied by PCR to determine the *cpe* genotype of the strain. Total DNA was isolated by using Advamax beads (Edge Biosystems, Gaithersburg, MD, USA) according to the manufacturer's instructions. IS elements downstream of *cpe* that determine the *cpe* genotype (IS*1151*-*cpe*, IS*1470*-like-*cpe*, or IS*1470*-*cpe*) of each isolate were characterized by using primers and protocols described in Table 1. The location of *cpe* (plasmid or chromosome) was concluded according to the genotyping results (Table 1).

C. perfringens isolates were then typed by PFGE with *Apa*I and *Sma*I (New England Biolabs, Beverly, MA, USA) to study the genetic relationships between isolates (*25*). PFGE profiles were analyzed visually and with a computer software program (Bionumerics, version 4.5; Applied Maths, Kortrijk, Belgium). The similarities between macrorestriction patterns (MRP) were expressed by Dice coefficient correlation, and clustering by the unweighted pair-group method with arithmetic averages was used to construct a dendrogram.

Cytotoxicity Test on Vero Cells

To test the capability of *cpe*+ strains to produce CPE, 1–3 *cpe*+ isolates representing each MRP were sporulated (*26*). The final culture in modified Duncan-Strong medium (Sigma-Aldrich Chemie, Steinheim, Switzerland) was examined by phase-contrast microscopy to confirm the sporulation of the strain. The culture was then sonicated until >95% of the spores were free, as determined by phase-contrast microscopy. The culture was centrifuged at 1,500× *g* for 25 min at 4°C, and cytotoxicity of the supernatant was tested with a Vero cell assay according to Sandvig and Olsnes (*27*). The assay monitors the inhibition of protein synthesis in Vero cells after addition of toxic proteins, including CPE. The Vero cells were grown in a minimal essential medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum.

The supernatant was precipitated in 80% saturated ammonium sulfate and kept at 4°C before the Vero cell assay. The strain was defined as producing CPE when the inhibition of protein synthesis of Vero cells was >20%.

Results

Detection and Isolation

The gene encoding for *cpe* was detected in the feces of 25 food handlers (18%). For 23 samples (92%), the heated tube showed the positive result, and for 3 of them the unheated tube also yielded a positive PCR result. For 2 samples, only the unheated tube showed a positive PCR result. No association between gender or age and carrier status of *cpe* was found (Table 2).

The HGMF-CH method was used to determine whether *cpe*+ *C. perfringens* was present in 23 persons; isolation was successful from 11 persons. The average number of *cpe*+ *C. perfringens* isolates carried by these persons was 2.5×10^2 CFU/g. In 10 of these persons, *cpe*– *C. perfringens* was also recovered; the average number was 1.8 ×

Table 1. Primers and PCR protocols for determining the genotype and location of *cpe**

Primer	Sequence (5′ to 3′)	Primer target	Sequence ref, GenBank accession no.	Ref	Size	*cpe* genotype	*cpe* location
CPEmmF	CAAGTCAAATTCTTAATCCT	*cpe*	(*22*), Y16009	(*12*)	1.2 kb	IS*1151-cpe*	Plasmid
1151 R	CATGGCCGTCAACCTAAGAAG	IS*1151*	(*23*), X60694				
CPEmmF	CAAGTCAAATTCTTAATCCT	*cpe*	(*22*), Y16009	(*12*)	2.4 kb	IS*1470-cpe*	Chromosome
*1470*mR	TGAAAACCGTGAAGAATTTGG	IS*1470*	(*12*), X71844				
cpe4F	TTAGAACAGTCCTTAGGTGATGG AG	*cpe*	(*14*), AF511071	(*24*)	1.6 kb	IS*1470*-like-*cpe*	Plasmid
IS*1470*-likeR1.6	CTTTGTGTACACAGCTTCGCCAA TGTC	IS*1470*-like	(*24*), AF416450				

*Ref, reference.

10^4 CFU/g. In each of these 10 case-patients, *cpe*– *C. perfringens* formed a 10- to 1,000-fold majority of the *C. perfringens* population. In 1 person, only *cpe*+ *C. perfringens* isolates were recovered. A total of 77 *C. perfringens* isolates (average of 7 isolates per person) were recovered; all possessed the gene encoding for the α toxin only, which signified that they belonged to type A (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12 no11/06-0478_appT.htm). Of these, 36 isolates were positive for *cpe* (Appendix Table).

Molecular Typing

When the relative prevalence of different *cpe* genotypes was determined with PCR, strains representing the IS*1151-cpe* type were found in 5 persons (3.7%) and strains representing the IS*1470*-like-*cpe* type were found in 4 persons (2.9%), findings that indicated that all of these persons carried *C. perfringens* strains with plasmidially located *cpe*. A strain with chromosomally located *cpe*, representing the IS*1470-cpe* type, was detected in 1 person (0.7%). Furthermore, *cpe*+ *C. perfringens* strains representing none of the aforementioned types, referred to as *cpe*+ strains with an unknown genetic arrangement downstream of *cpe*, were observed in 2 persons (1.5%) (Appendix Table) (Figure 1).

In PFGE analysis, all isolates were typeable with *Sma*I restriction enzyme, and all but 2 isolates were typeable with *Apa*I. The discriminatory power was equal with both enzymes used, which showed a high genetic diversity among *C. perfringens*. PFGE analysis showed 1–5 different MRPs in each person, with *cpe*+ and *cpe*– isolates generally being unrelated to each other. However, in 1 case, 2 isolates (CPI 57–1 and CPI 57–2) from the same person showed identical MRPs with both restriction enzymes, the former being *cpe*+ and the latter *cpe*– (Appendix Table; Figure 2). In 8 of 11 persons, *cpe*+ isolates with similar MRPs were identified, whereas 3 persons carried *cpe*+ isolates with 2 different MRPs. In 2 of these persons (numbers 18 and 57), both MRPs represented the same *cpe* genotype (IS*1470*-like-*cpe*); the third person (number 75) had MRPs with different *cpe* genotypes (IS*1470*-like-*cpe* and IS*1151-cpe)* (Appendix Table).

Table 2. Association of sex and age with *cpe* in feces of healthy humans

Characteristic	*cpe* detected by PCR/ total no. fecal samples (%)
Sex	
Female	19/102 (18.6)
Male	6/34 (17.6)
Total	25/136 (18.4)
Age, y	
<20	6/40 (15.0)
20–50	16/79 (20.3)
>50	3/17 (17.6)
Total	25/136 (18.4)

Cytotoxicity Test on Vero Cells

Successful sporulation was achieved with isolates representing 9 different MRPs. In 8 (89%) of these MRPs, ≥20% inhibition of protein synthesis was detected in Vero cells, which suggested CPE production of the strain (Table 3).

Discussion

We report the first in-depth study of the fecal carriage of *cpe*+ *C. perfringens* by healthy humans, which showed that the organism is widely distributed in this ecologic niche. By using the novel HGMF-CH method, we demonstrated that low numbers of *cpe*+ *C. perfringens* strains are frequently present among the dominant *cpe*– *C. perfringens*. HGMF-CH proved invaluable in the isolation of *cpe*+ *C. perfringens*; in all instances, when both *cpe*+ and *cpe*– *C. perfringens* were isolated, the former existed as a minority and thus would have been missed by conventional isolation methods.

Healthy persons are a rich reservoir for *cpe*+ *C. perfringens;* type A strains representing several different *cpe* genotypes (strains with plasmidially located IS*1470*-like–*cpe* or IS*1151-cpe* and chromosomally located IS*1470-cpe*) as well as type A strains with unrecognized genetic arrangement attached to *cpe* were present. That IS*1151-cpe* was the most prevalent and IS*1470-cpe* represented the minority of the *cpe* genotypes are findings in

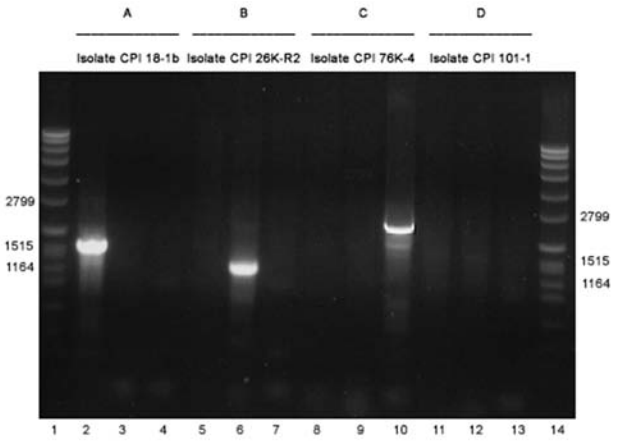


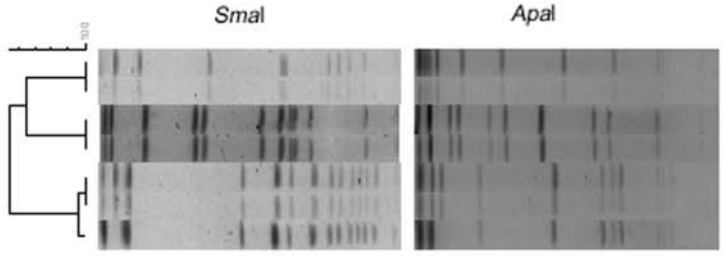
Figure 1. PCR analysis determining the genotype of enterotoxin gene-carrying *Clostridium perfringens* type A isolates obtained from healthy persons. All 4 strains are studied with 3 different primer sets, described in Table 1. For A, B, C and D, the isolate represents genotype IS*1470*-like-*cpe*, IS*1151*-*cpe*, IS*1470*-*cpe*, or unknown genotype, respectively.

line with a previous study that suggested that *cpe*+ *C. perfringens* strains with plasmidially located *cpe* are more common in nature than *cpe*+ *C. perfringens* strains with chromosomally located *cpe* (*28*). The presence of strains with unrecognized genetic arrangement attached to *cpe* reflects the wide genetic variety of *cpe*+ strains. Because production of CPE was demonstrated in 1 of these *cpe*+ strains with an unknown *cpe* genotype, the gene was apparently intact and functional.

The presence of *cpe*+ *C. perfringens* type A strain with chromosomally located *cpe* and a full capacity to produce CPE in the feces of healthy food handlers indicates that human handling of food should be considered a risk factor for contamination. *C. perfringens* type A food poisoning typically follows from the ingestion of *cpe*+ *C. perfringens* vegetative cells formed in food during storage and serving (*29*). The low numbers of *cpe*+ *C. perfringens* spores present in the feces of the person handling the food may be transferred to the food. Under favorable conditions, *cpe*+ strains with chromosomally located *cpe* will easily survive and multiply during food processing and cause food poisoning because they survive broader temperature ranges during growth and maintenance phases than other *C. perfringens* strains (*15*,*30*).

Evidence of in vivo horizontal transfer of *cpe* between *C. perfringens* strains was obtained in the PFGE analysis. First, in 2 persons (numbers 18 and 57), IS*1470*-like-*cpe* was observed in strains with no genetic relatedness, which indicated lateral spread of IS*1470*-like-*cpe* (Appendix Table). In vitro conjugative transfer of the *cpe* plasmid has been demonstrated with strain F4969 carrying IS*1470*-like-*cpe* (*18*,*24*), which supports our findings. Second, the evidence of in vivo horizontal transfer of *cpe* was further strengthened by observing a loss or acquisition of IS*1470*-like-*cpe* in 1 strain (MRP 15) (Appendix Table). In this case, the isolate CPI 57–1 carried IS*1470*-like-*cpe*, whereas CPI 57–2 was lacking in the same element; these strains were nevertheless isolated from the same person and shared an identical MRP. A potential donor or recipient strain (MRP 13 and 13b) was demonstrated from the same sample, which carried IS*1470*-like-*cpe* but shared no genetic relatedness to CPI 57–1 (Appendix Table) (Figure 2). All IS*1151*-*cpe* isolates from the same persons shared identical MRPs; thus, no evidence for in vivo lateral transfer of IS*1151*-*cpe* was observed.

Finally, our study provides a new insight into the pathogenesis of AAD and SD caused by *cpe*+ *C. perfringens* type A. These diseases have been speculated to result from the ingestion of small numbers of *cpe*+ strains, which transfer the *cpe* plasmid to *cpe*– *C. perfringens* strains present in the normal intestinal microbiota (*17*). Our results support this theory because we found small numbers of *cpe*+ *C. perfringens* type A with plasmidially located *cpe* in the human gastrointestinal tract as well as evidence for the lateral spread of *cpe*. Our findings therefore indicate that AAD or SD caused by *cpe*+ *C. perfringens* type A may occur as an endogenous infection, present in the gastrointestinal tract and causing the disease after exposure to antimicrobial drugs or other predisposing factors. However, the capacity of these *cpe*+ strains to persist in the gastrointestinal tract remains unknown; as does how these strains find their way to the gastrointestinal tract. Nevertheless, because they are apparently common in human feces, *cpe*+ strains are presumably acquired from



Isolate	*cpe*	MRP	*cpe* genotype	*cpe* location
CPI 57 K-2	-	14	ND	ND
CPI 57 K-3	-	14	ND	ND
CPI 57-1	+	15	IS 1470-like-cpe	Plasmid
CPI 57-2	-	15	ND	ND
CPI 57 K-1	+	13	IS 1470-like-cpe	Plasmid
CPI 57 K-1b	+	13	IS 1470-like-cpe	Plasmid
CPI 57 K-4	+	13b	IS 1470-like-cpe	Plasmid

Figure 2. Pulsed-field gel electrophoresis analysis and determination of the *cpe* genotype of *Clostridium perfringens* isolates obtained from a healthy person. ND, not determined.

Table 3. Cytotoxicity test on Vero cells to determine the production of CPE by *cpe+ C. perfringens* strains obtained from healthy persons

Isolate	MRP*	CPE†	*cpe* genotype	*cpe* location
CPI 18–3	1	+	IS*1470*-like-*cpe*	Plasmid
CPI 18–2	2	NS	IS*1470*-like-*cpe*	Plasmid
CPI 26K-R3p	5	+	IS*1151-cpe*	Plasmid
CPI 39–1b, CPI 39K-7	6	+	IS*1470*-like-*cpe*	Plasmid
CPI 44K-R3, CPI 44K-R7	10	NS	Untypeable	Not known
CPI 53K-R3	11	NS	IS*1151-cpe*	Plasmid
CPI 57K-1	13	+	IS*1470*-like-*cpe*	Plasmid
CPI 57–1	15	NS	IS*1470*-like-*cpe*	Plasmid
CPI 63K-R5	19	NS	IS*1151-cpe*	Plasmid
CPI 75–3a, CPI 75–3b, CPI 75–5b	21	-	IS*1151-cpe*	Plasmid
CPI 75–4	22	+	IS*1470*-like-*cpe*	Plasmid
CPI 76K-4, CPI 76K-5	24	+	IS*1470-cpe*	Chromosome
CPI 101–4	25	+	Untypeable	Not known
CPI 103K-3, CPI 103K-5, CPI 103K-6	29	+	IS*1151-cpe*	Plasmid

*MRP, macro restriction pattern.
†+, present; –, absent, NS, not sporulated and thus cytotoxicity test on Vero cells not performed.

the environment. The question then arises whether these *cpe+* strains are ingested with food, which would indicate that AAD and SD caused by *cpe+ C. perfringens* type A are foodborne.

In conclusion, healthy humans serve as a rich reservoir for *cpe+ C. perfringens* type A strains and may play an important role in gastrointestinal diseases caused by this pathogen. Humans should therefore be considered a risk factor for spread of *C. perfringens* type A food poisoning. Future studies to determine the presence of different *cpe* genotypes in other ecologic niches are warranted to elucidate the epidemiology of this major pathogen.

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Clostridium difficile PCR Ribotypes in Calves, Canada

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We investigated *Clostridium difficile* in calves and the similarity between bovine and human *C. difficile* PCR ribotypes by conducting a case-control study of calves from 102 dairy farms in Canada. Fecal samples from 144 calves with diarrhea and 134 control calves were cultured for *C. difficile* and tested with an ELISA for *C. difficile* toxins A and B. *C. difficile* was isolated from 31 of 278 calves: 11 (7.6%) of 144 with diarrhea and 20 (14.9%) of 134 controls (p = 0.009). Toxins were detected in calf feces from 58 (56.8%) of 102 farms, 57 (39.6%) of 144 calves with diarrhea, and 28 (20.9%) of 134 controls (p = 0.0002). PCR ribotyping of 31 isolates showed 8 distinct patterns; 7 have been identified in humans, 2 of which have been associated with outbreaks of severe disease (PCR types 017 and 027). *C. difficile* may be associated with calf diarrhea, and cattle may be reservoirs of *C. difficile* for humans.

Clostridium difficile, a gram-positive, spore-forming, anaerobic bacterium, has been associated with pseudomembranous colitis and nosocomial and antimicrobial drug–associated diarrhea in humans (*1*). Recently, research has suggested that the frequency, severity, and relapse of *C. difficile*–associated disease (CDAD) are increasing in Europe and North America (*1*,*2*). The most common risk factor for CDAD in humans is the use of antimicrobial drugs, particularly fluoroquinolones (*3*–*5*). Of recent concern, hypervirulent *C. difficile* strains have been associated with outbreaks of severe CDAD (*2*,*6*).

The pathophysiology of CDAD involves colonization of the intestinal tract with *C. difficile* and production of its toxins (*7*–*9*). At least 3 cytotoxins are currently described for *C. difficile*: toxins A and B (glucosyltransfersases) and a binary toxin (CDT, ADP-ribosyltransferase) (*10*). Toxins TcdA and TcdB are encoded by 2 separate genes, *tcdA* and *tcdB*, located in a 19.6-kb pathogenicity locus (PaLoc). The expression of these 2 genes is regulated by a putative negative regulator within PaLoc, the *tcdC* gene (*11*). Deletions in *tcdC* are believed to result in overexpression of *tcdA* and *tcdB* and increased production of toxins A and B, which may account for the apparent higher pathogenicity in certain ribotypes (i.e., PCR type 027) (*1*). Some strains also produce binary toxin, which is encoded by the genes *cdtA* and *cdtB* located outside PaLoc (*10*). The role of binary toxin in disease is currently under investigation (*12*). Isolates producing ≥1 of these toxins (A, B, or binary) are currently referred to as toxigenic strains (*10*). *C. difficile* also appears to be an important cause of enteric disease in other species, including horses, dogs, and pigs (*7*,*8*,*13*,*14*).

Neonatal calf diarrhea (NCD) is a common cause of illness (10.2%) and death in preweaning calves (*15*). A variety of enteropathogens have been implicated in NCD; however, many cases are currently idiopathic (*16*). Although *C. difficile* infection has been suggested as a cause of diarrhea and enteritis in calves (*17*), further published evidence is lacking. The objectives of this study were to evaluate the role of *C. difficile* in NCD, genotypically and phenotypically characterize isolates from calves, and compare calf and human isolates.

Materials and Methods

Farms and Calves

A total of 102 dairy farms in southern Ontario, Canada, were included in the study. Farms were visited from May through September 2004 to obtain 1 fecal sample from calves <1 month of age. Fecal samples (>4 g) were obtained from 10 consecutively born calves per farm.

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Samples were scored at the farm using a 4-grade fecal scoring system and then stored at 4°C within 6 hours of collection. A score of 1 represented hard, dry fecal matter; score 2, pasty and sticky feces; score 3 soft feces; and score 4, watery feces that would adopt the shape of the container immediately after sampling. Samples with a score of 4 were considered to have diarrhea, whereas scores of 1 and 2 were controls. Samples with a score of 3 were discarded to reduce selection bias. Selected samples were recoded for blinding purposes and stored at –70°C within 24 hours of collection. A questionnaire that requested information about colostrum quality and administration, diet, housing, cleaning and disinfection practices, antimicrobial or antiprotozoal feed supplements, level of nose-to-nose contact among calves, vaccination of dams, and dehorning was administered on each farm to investigate risk factors for *C. difficile* in feces.

C. difficile Culture and Detection of Toxins A and B

Fecal samples were processed within 2 hours after thawing. Enrichment culture was performed as previously described (*7*,*18*). Briefly, ≈1 g of homogenized fecal matter was mixed with 2 mL of 96% ethanol and agitated at room temperature for 50 minutes to select for bacterial spores. The sediment was recovered after centrifugation at 3,800 × *g* for 10 minutes and resuspended in 5 mL of cycloserine-cefoxitin fructose broth (*C. difficile* agar and *C difficile* supplement SR0096; Oxoid, Columbia, MD, USA) that was incubated anaerobically at 37°C for 7 days. This broth was treated with 96% ethanol (1:1 vol/vol), centrifuged at 3,800 × *g* for 10 minutes, and the sediment was resuspended in 200 μL of sterile deionized water. Thereafter, 200 μL of sediment was streaked onto cycloserine-cefoxitin fructose agar and blood agar that were incubated anaerobically at 37°C. Plates were evaluated in an anaerobic environment daily for ≤5 days. If present, at least 2 colonies (swarming, flat, rough, nonhemolytic) were subcultured. *C. difficile* was identified by Gram stain (spore-forming gram-positive rods) and detection of L-proline aminopeptidase activity (Pro Disc, Remel, Lenexa, KS, USA) (*19*). Isolates were stored at –70°C until molecular analyses were performed.

Feces were screened for *C. difficile* toxins A and B by using an ELISA (Tox A/B ELISA, TechLab, Blacksburg, VA, USA) (*20*). The test was performed per manufacturer's instructions. Two observers interpreted the reactions in a blinded fashion.

Extraction of DNA

DNA was extracted by using a Chelex resin-based kit (InstaGene Matrix, Bio-Rad, Laboratories, Hercules, CA, USA) (*21*). After centrifugation of the *C. difficile* DNA–containing solutions, 125 μL of supernatant was collected and stored at –20°C as a template for PCR analyses.

PCR Ribotyping

PCR ribotyping analyses were performed as previously described (*22*). DNA was amplified by using a thermal cycler (Touchgene Gradient, Techne Inc., Burlington, NJ, USA). Ribotype patterns were compared visually with *C. difficile* PCR ribotypes from humans and other animals from the provinces of Ontario, Quebec, and Manitoba, Canada. The first isolate identified for each PCR ribotype was submitted to the Anaerobe Reference Laboratory, University Hospital of Wales, Cardiff, United Kingdom, for comparison (*23*).

Detection of *tcdA*, *tcdB*, *tcdC*, and *cdtB* Genes

Amplification of nonrepeating and repeating sequences of the *tcdA* gene and the nonrepeating sequences of the *tcdB* gene was performed as previously described (*24*). Identification of *tcdC* and *cdtB* genes was based on previous protocols (*11*,*24*,*25*). Reference strains were included as positive and negative controls in every experiment.

Antimicrobial Drug Susceptibility Tests

MICs for metronidazole, clindamycin, levofloxacin, and vancomycin were determined by using the E-test method (AB Biodisk, Solna, Sweden) (*26*). A McFarland standard 1 suspension of pure *C. difficile* colonies was placed on Muller-Hinton blood agar plates (Oxoid, Basingstoke, UK). After 48 hours of anaerobic incubation, MICs were determined by consensus of 2 observers.

Toxinotyping of *C. difficile* Strains

Toxinotyping analysis involved amplification and enzymatic restriction of PCR fragment A3 of *tcdA* and PCR fragment B1 of *tcdB*. This was performed following a previously published protocol (*27*).

Other Enteropathogens

Because intestinal cryptosporidiosis was common (40.6%) in dairy calves <28 days of age in the study area (*16*), samples examined for *C. difficile* were also tested for *Cryptosporidium* spp. oocysts (sucrose wet mount test) to control for potential interactions regarding diarrhea. Other calf enteropathogens were not investigated because they are less prevalent in the region (L.A. Trotz-Williams et al., unpub. data).

Statistical Analysis

Multivariate stepwise logistic regression analyses were performed by using SAS statistical software (SAS Institute, Cary, NC, USA). Associations between farm management data, age, sampling month, and results from

laboratory tests were investigated by using a generalized model procedure (GenMod in SAS). Variables associated with diarrhea and *C. difficile* or its toxins in feces were investigated. During initial model building, variables with p<0.15 were selected to construct final models. Parameters were considered statistically significant if p values were <0.05. A generalized linear mixed model controlling for farm as a random effect was used to estimate and test the farm variance component. Relationships between *C. difficile* toxins and diarrhea and between *C. difficile* toxins and the age and month of sampling were investigated in the models. Pairwise comparisons of least square means were performed, and approximated Tukey adjusted p values were computed. Reported exact p values, odd ratios (ORs), and 95% confidence intervals (CIs) were determined with exact conditional logistic regression tests by using LogXact 5 software (Cytel Inc., Cambridge, MA, USA) when analyses did not yield exact values with SAS software.

Results

A total of 278 calves were studied: 144 with diarrhea and 134 controls. The mean age of the sample was 14.2 days (range 5–30 days); 39 calves were 5–7 days of age, 107 were 8–14 days of age, 96 were 15–21 days of age, and 32 were 22–30 days of age. Four calves had no age recorded and were not used for descriptive information regarding age. The mean ages of the control calves (14.8 days, 95% CI 13.7–15.9) and calves with diarrhea (13.9 days, 95% CI 13.0–14.7) were not significantly different (p = 0.16).

C. difficile was isolated from 31 (11.2%) of 278 calves from 25 (25%) of 102 farms. This bacterium was more commonly identified in feces from control calves (14.9%, 20/134) than in feces from calves with diarrhea (7.6%, 11/144) (OR 3.47, 95% CI 1.27–10.24, exact 2-tailed p = 0.009).

C. difficile toxins A and B were identified in feces of 85 (30.6%) of 278 calves from 58 (56.8%) of 102 farms: 57 (39.6%) of 144 calves with diarrhea and 28 (20.9%) of 134 controls (OR 3.07, 95% CI 1.62–5.96, exact 2-tailed p = 0.0002). *C. difficile* and its toxins were detected concurrently in only 6 (4.2%) of 144 calves with diarrhea and in 7 (5.2%) of 134 controls.

Generalized linear mixed model analysis with farm as a random effect showed no farm variance component (coefficient 0). Thus, farm was included in subsequent models as a fixed effect. Generalized linear model analyses showed that none of the farm management practices surveyed were associated with diarrhea or *C. difficile* test results. Conversely, the month of sampling (p = 0.008) and the age of the calves (p = 0.005) were significant variables when modeling for the ELISA result as the outcome. May, June, and July were associated with higher ORs of yielding a positive fecal *C. difficile* toxin test result than was August (Table 1).

Table 1. Estimated odds ratios for a calf to have a positive toxin A/B ELISA result, southern Ontario, Canada, 2004*

Pairwise comparison	Odds ratio	95% CI	Adjusted Tukey p values
May vs August†	3.62	1.8–8.3	0.007
June vs August†	3.17	1.3–7.7	0.029
July vs August†	2.58	1.2–5.5	0.038
May vs July	1.41	0.7–2.8	0.59
June vs July	1.23	0.6–2.6	0.85
May vs June	1.14	0.5–2.6	0.95

*CI, confidence interval. The number of positive toxin A/B ELISA test results and calves sampled per month was May, 23/57; June, 16/41; July, 34/105; and August, 12/75. August data include 2 observations from September.
†Statistically significant.

When the association with age was analyzed, a linear relationship was found between age of calves and probability of a positive test result for *C. difficile* toxins. Fecal samples from older calves were less likely than samples from younger calves to be positive for *C. difficile* toxins; the estimated OR was 2.0 for every 10 days of age difference at any point from 5 and 30 days of age (natural antilogarithm of [0.0691 × no. of days of interest]; 95% CI 1.22–3.24, p = 0.006). No association was found between administration of feed supplemented with oxytetracycline (33 calves on 11 farms) or anticoccidial drugs (251 calves on 91 farms) and *C. difficile* and its toxins in feces.

Cryptosporidium spp. oocysts in feces were significantly associated with diarrhea and identified in 80 (55.9%) of 144 calves with diarrhea and 19 (14.2%) of 134 control calves (OR 8.23, 95% CI 4.35–16.26, exact 2-tailed p<0.0001). However, generalized linear model analysis showed no interaction between *Cryptosporidium* spp. and *C. difficile* toxins (p>0.5) or between *Cryptosporidium* spp. and *C. difficile* culture (p>0.58).

Eight calf PCR ribotypes were identified among 31 *C. difficile* isolates (Figure). Of these, 7 ribotypes represented by 30 (96.7%) isolates were toxigenic (Table 2). Isolates from 5 ribotypes had the classic *tcdC* fragment, and ribotypes A11 and F12 had the major type A deletion (≈39 bp deletion) (Table 2). Isolates of ribotype D189 had a *tcdC* fragment, which is indicative of either a type B or C deletion (≈18 bp).

The MIC range, MIC_{50}, MIC_{90}, and the percentage of resistant *C. difficile* isolates are shown in Table 3. All 30 isolates tested were susceptible to metronidazole and vancomycin. The prevalence of resistance for clindamycin and levofloxacin was similar (73%, 22/30 isolates), but 18 (82%) of the 22 resistant isolates were resistant to both antimicrobial drugs. Calf ribotypes A11 (5/9 isolates), B89 (9/9), C129 (2/2), and D189 (4/4) were overrepresented among the resistant isolates.

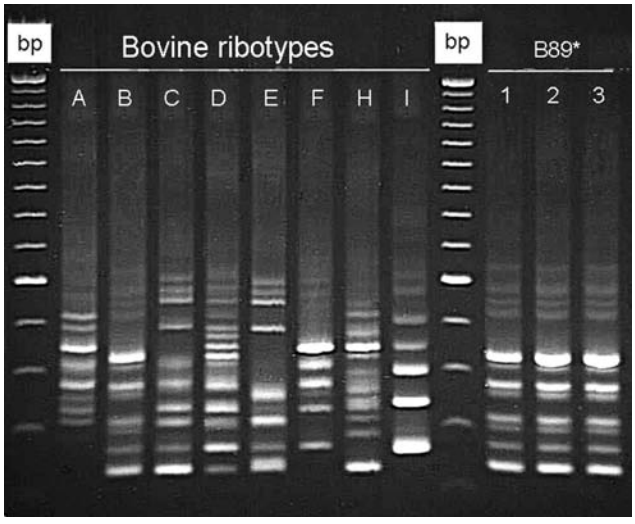

Figure. *Clostridium difficile* PCR ribotypes of bovine origin (dairy calves), Ontario, Canada, 2004. *Calf isolate classified as PCR ribotype 017 at the Anaerobe Reference Laboratory, University Hospital of Wales, Cardiff, United Kingdom. Isolates of human (lane 1), calf (lane 2), and canine (lane 3) origin identified in Ontario are indistinguishable. The first and tenth wells contain 100-bp molecular mass markers.

Comparison of the 8 calf PCR ribotypes with a local collection of 25 ribotypes of *C. difficile* isolated from humans showed that 3 calf ribotypes representing 17 (54.8%) of 31 isolates were indistinguishable (calf ribotypes B89 and D189) or similar (calf ribotype C129) to ribotypes associated with CDAD in humans in Ontario and Quebec (Figure). Ribotype B89, a strain that produces toxin B but not toxin A, was indistinguishable from a strain obtained from patients during a nosocomial outbreak of CDAD in Manitoba, Canada (Figure) (*28*). When compared with a collection of canine isolates from southern Ontario (*29*), this ribotype was also identified in healthy dogs (Figure). Isolates B89 and D89 were not clustered; they were isolated from farms distributed across the studied region with ≈500 km between the most distant ones. Comparison of 7/8 calf ribotypes (representing 30/31 isolates) with a *C. difficile* PCR ribotype library at the Anaerobe Reference Laboratory, University Hospital of Wales, Cardiff, United Kingdom, that contained >160 *C. difficile* ribotypes showed that all bovine ribotypes have been identified in humans (Table 2). Toxinotyping of isolates from calf ribotypes B89/ARL-UK PCR ribotype 017 and D189/ARL-UK PCR type 027 indicated that they were toxinotypes VIII and III, respectively. Other calf ribotypes were not toxinotyped.

Discussion

This study has demonstrated that shedding of *C. difficile* is common in dairy calves in Ontario regardless whether they have enteric disease. The overall prevalence of shedding (11.2%) was similar to that previously reported (*17*). However, that shedding of *C. difficile* was more common in control animals was surprising, particularly because 96.7% of the isolates were toxigenic. The reason for this finding is unclear, and natural and methodologic reasons should be considered. Whether the isolation method used in this study resulted in identification bias in favor of 1 of the groups (i.e., control animals) is not known. Pretreatment of fecal samples with ethanol has been shown to facilitate the recovery of *C. difficile* in asymptomatic humans (*18*). However, how this method would work in calves with and without diarrhea is unknown. The dilutional effect of watery stools could have prevented *C. difficile* from being isolated from calves with diarrhea, or *C. difficile* may not be a primary pathogen in calves. In addition, the concentration of *C. difficile* in the

Table 2. *Clostridium difficile* PCR ribotypes and toxin genes of 31 isolates obtained from dairy calves in southern Ontario, Canada, 2004

Toxigenic classification†	Calf PCR ribotypes*								Subtotal, no. (%)
	A-11	B-89	C-129	D-189	E-257	F-12	H-75	I-157	
A–B$^-$, *cdtB*$^-$	–	–	–	–	–	1	–	–	1 (3.2)
A–B$^-$, *cdtB*$^+$	–	–	–	–	–	2	–	–	2 (6.5)
A+B$^+$, *cdtB*$^-$	–	–	2	–	4	–	1	1	8 (25.8)
A+B$^+$, *cdtB*$^+$	7	–	–	4	–	–	–	–	11 (35.5)
A–B$^+$, *cdtB*$^-$	–	8	–	–	–	–	–	–	8 (25.8)
A–B$^+$, *cdtB*$^+$	–	1	–	–	–	–	–	–	1 (3.2)
Subtotal, no. (%)	7 (22.6)	9 (29)	2 (6.5)	4 (12.9)	4 (12.9)	3 (9.7)	1 (3.2)	1 (3.2)	31 (100)
Type of *tcdC* deletion‡, (no.)	A (7)	None (9)	None (2)	B (4)	None (4)	A (3)	None (1)	None (1)	
Human PCR ribotypes§	078	017	077	027	014	033	078	NS¶	

*By PCR typing method of Bidet et al. (*22*).
†A, toxic gene *tcdA*; B, *tcdB*; – , absence or presence of the categorized gene; *cdtB*, gene that codifies CDTb, the binding segment of the binary toxin.
‡None, *tcdC* with no deletions (≈345 bp); A, type A deletion of ≈39 bp; B, nondifferentiated type B or C deletion of ≈18 bp (*11*).
§Selected isolates processed at the Anaerobe Reference Laboratory, University Hospital of Wales, Cardiff, UK (*23*).
¶NS, not submitted for analysis.

Table 3. MIC_{50} and MIC_{90} range and resistance frequencies of 30 bovine *Clostridium difficile* isolates to 4 antimicrobial drugs by E-test on Muller-Hinton agar after 48 h of incubation*

Drug	MIC_{50}, μg/mL	MIC_{90}, μg/mL	Range, μg/mL	Resistant isolates, % (no. resistant/no. tested), MIC (μg/mL)	Overrepresented PCR ribotypes (no.)
Vancomycin	0.5	0.75	0.25–1.5	0	0
Metronidazole	0.38	0.75	0.125–2.0	0	0
Levofloxacin	32	32	4 to >32	73 (22/30), ≥32	B89 (9/9), C129 (2/2), D189 (4/4), other 3 ribotypes (7 isolates)
Clindamycin	16.0	>256	6 to >256	73 (22/30), ≥12; 37 (11/30), ≥256	B89 (9/9), C129 (2/2)

*The breakpoints used were vancomycin susceptible, ≤4.0 μg/mL; vancomycin resistant, >32.0 μg/mL; metronidazole susceptible, ≤8.0 μg/mL; metronidazole resistant, >32.0 μg/mL; clindamycin susceptible, ≤2.0 μg/mL; clindamycin resistant >8.0 μg/mL; levofloxacin susceptible, ≤2.0 μg/mL; levofloxacin resistant, >8.0 μg/mL.

intestinal tract may not correlate with the concentration of spores in feces. Since quantitative culture was not performed in this study, conclusions cannot be made.

The pathophysiology and epidemiology of *C. difficile* are not completely understood in humans, and some studies have reported that asymptomatic colonization with *C. difficile* may have a protective effect against CDAD (*30*). In humans, 50%–80% of asymptomatic infants may be colonized with toxigenic *C. difficile* and have its toxins in their feces (*31*). *C. difficile* has been reported to affect neonatal foals and piglets (*7*,*8*).

Detection of toxins A and B in feces of humans with diarrhea is considered diagnostic for CDAD (*31*,*32*). The positive association between fecal *C. difficile* toxins and calf diarrhea found in our study indicates that *C. difficile* might be a pathogen in calves. However, the clinical relevance of this association is uncertain because it is based on the assumption that the ELISA used has acceptable sensitivity and specificity in calves. The validity of this ELISA has not been reported for most animal species, including cattle. For humans and piglets, adequate sensitivities and specificities for this ELISA (65%–95% and 95%–100%, respectively) (*20*,*32*,*33*) contrast with recently reported suboptimal performance for canine feces (*34*). With an apparent interspecies variability of the ELISA, validation of this test for bovine feces is required before conclusions regarding causal associations can be made.

The finding that calves were more likely to have detectable levels of *C. difficile* toxins in their feces early in life is consistent with findings of a previous study (*17*). The reason for this is unclear, although *C. difficile* may be better able to colonize, proliferate, and produce toxins in younger animals with less developed intestinal microflora. In other animal species and humans, administration of antimicrobial drugs is considered a predisposing factor for development of CDAD (*3*,*7*,*35*,*36*). No statistical associations were identified in this regard at the calf level because questionnaires were designed to explore farm practices.

Molecular analyses showed that a relevant proportion of the *C. difficile* isolates (9/31) had *tcdB* genes but not *tcdA* genes ($A–B^+$). These variant isolates are uncommon in humans but have been reported in association with outbreaks of CDAD (*2*,*36*). In a previous study in calves, no $A–B^+$ isolates were identified (*17*). This discrepancy could be due to potential differences between the 2 study populations.

In our study, the 9 calf $A–B^+$ isolates and a control strain were classified as ribotype pattern B89 type 017 (Figure). This ribotype has been reported in outbreaks of CDAD in humans in various countries (*2*,*28*,*36*), including the Canadian provinces of Ontario, Quebec, and Manitoba, from which the human control strain was obtained (*28*). Toxinotyping (type VIII) and *tcdC* analysis (classic gene) of these 9 calf isolates supported their similarity to human strains. The epidemiologic explanation for the presence of this human epidemic strain in calves and in healthy dogs (*29*) is uncertain, but this finding raises the concern of potential animal-to-human transmission and vice versa. No isolates of bovine origin were available for additional retrospective comparisons.

The second major calf ribotype common to humans in Ontario and Quebec was D189/PCR ribotype 027 (positive for *tcdA*, *tcdB* and *cdtB*, type B *tcdC* deletion, and toxinotype III). Molecular characteristics of this ribotype indicate that it is a hypertoxin-producing ribotype recently reported as a cause of serious outbreaks of disease in humans in North America and Europe (*1*). In Quebec, Canada, *C. difficile* type 027 was isolated during an outbreak from 67% of persons with hospital-acquired CDAD and 37% of persons with community-acquired CDAD (*1*). The pathogenicity of this ribotype is believed to be associated with a high production of toxins A and B in vitro, and with fluoroquinolone resistance (*3*,*4*).

The 4 calf isolates of PCR D189/ribotype 027 identified in our study were not geographically clustered. This result and the recent finding of this strain in a dog in Ontario indicate that this *C. difficile* ribotype may be widely disseminated in the community in different animal species (*37*). The public health consequences of this are unclear and require further study. Whether cattle could play a role in dissemination of this strain through direct contact, environmental contamination, or the food chain should be determined. Although *C. difficile* is not considered a foodborne pathogen, it has been identified in raw meat intended for pet

consumption (*38*) and in retail meat from grocery stores in Ontario (A. Rodriguez-Palacios et al., unpub. data).

Results of antimicrobial drug susceptibility tests for metronidazole, vancomycin, and clindamycin are consistent with those of previous reports in humans, in which antimicrobial susceptibility of *C. difficile* strains to metronidazole and vancomycin was ≈100% and antimicrobial resistance to clindamycin was ≈70%–80% (*2*,*26*,*35*). Most isolates (73%) were resistant to levofloxacin, which is not administered to cattle. Antimicrobial drug resistance to fluoroquinolones has been described in *C. difficile* PCR ribotype 027 as a major risk factor for development of CDAD (*4*,*5*). The development of fluoroquinolone resistance in human-derived strains has been hypothesized to result from increased use of these antimicrobial drugs, which has also been associated with a higher risk for CDAD in hospitals (*3*,*5*).

Use of fluoroquinolones was not voluntarily reported for any of the farms or calves in this study, and levofloxacin resistance cannot be extrapolated to other fluoroquinolones (*39*). In Canada, fluoroquinolones are not approved for use in dairy cattle or veal calves. Fluoroquinolones have not been approved for veterinary use in any food-producing animals in Canada until recently, when a commercial enrofloxacin product was approved only for use in beef cattle with unresponsive respiratory disease (*39*). As part of a Canadian surveillance program, Health Canada, through the Canadian Integrated Program for Antimicrobial Resistance Surveillance, has monitored fluoroquinolone resistance in strains of *Escherichia coli* and *Salmonella* spp. from beef cattle since 2001–2002. According to the Canadian Integrated Program for Antimicrobial Resistance Surveillance 2002 and 2003 reports, no resistance to fluoroquinolones has been observed (*40*). Thus, the source of fluoroquinolone resistance in calf-derived *C. difficile* isolates in our study is uncertain and is not substantiated on the hypothesis of excessive use of fluoroquinolones, i.e., enrofloxacin, in cattle. Whether this resistance has any epidemiologic association with companion animals (i.e., dogs) or humans for which fluoroquinolones have been approved for many years remains unknown.

The results of our study indicate that *C. difficile* may play a role in neonatal calf diarrhea, which is a serious concern in the bovine industry. Calf *C. difficile* isolates that are indistinguishable from human strains and have fluoroquinolone resistance and *tcdC* deletions also raise the possibility of interspecies transmission. Although this study did not confirm that infection with *C. difficile* is zoonotically transmitted, further study is indicated to evaluate this possibility. Investigations of recent changes in the epidemiology of CDAD and identification of new pathogenic genotypes should also involve concurrent evaluation of animal reservoirs or origins. Validation studies are also required to assess culture protocols and immunoassay tests for identification of *C. difficile* and its toxins in cattle feces.

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Dr Rodriguez-Palacios recently completed a doctorate degree in veterinary sciences and a residency in large animal internal medicine at the Ontario Veterinary College, University of Guelph. His research interests include development of probiotics for prevention of diarrhea and the epidemiology of infectious diseases of large animals, particularly of pathogens with potential public health implications.

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VEB-1 in *Achromobacter xylosoxidans* from Cystic Fibrosis Patient, France

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Multidrug-resistant *Achromobacter xylosoxidans* was recovered from the sputum of a patient with cystic fibrosis. The VEB-1 extended-spectrum β-lactamase was detected on a class 1 integron. This first report of a VEB-1–producing isolate in this population requires further investigation to determine its distribution.

Achromobacter (formerly *Alcaligenes) xylosoxidans* is a newly emerging microorganism isolated with increased frequency from the lungs of patients with cystic fibrosis (CF), but information about its clinical relevance is limited (*1*). *A. xylosoxidans* is innately resistant to many antimicrobial drugs (*2*), except piperacillin, piperacillin-tazobactam, and imipenem, and moderately susceptible to ceftazidime (45% of susceptible isolates), which is widely used to treat infection due to *Pseudomonas aeruginosa* (*3*,*4*). The mechanisms involved in cases of high-level resistance to ceftazidime have not been described for *A. xylosoxidans*. Possible mechanisms for ceftazidime resistance among gram-negative bacilli are alterations in outer membrane proteins, overproduction of cephalosporinase, or production of an extended-spectrum β-lactamase (ESBL). ESBLs are enzymes distributed worldwide (*5*) that hydrolyze oxyimino-cephalosporins and monobactams and are susceptible to β-lactamase inhibitors such as clavulanic acid and tazobactam. We report on the isolation from a CF patient of *A. xylosoxidans* that produced the VEB-1 ESBL. This is the first report of ESBL production in *A. xylosoxidans* and the first report of a VEB-1–producing isolate from a CF patient.

The Study

During the past 10 years in our 1,600-bed university hospital, 37 CF patients had ≥1 respiratory tract specimen that contained *A. xylosoxidans*. Preliminary pulsed-field gel electrophoresis of these strains has failed to identify shared isolates among the patients, but studies are ongoing. In November 2003, *A. xylosoxidans* 476 (AX476) was isolated from the sputum of a 17-year-old male CF patient. This patient had good pulmonary function (forced expiratory volume 1 = 99% of predicted value), had never been colonized or infected by *P. aeruginosa*, and therefore never received ceftazidime. The strain was identified with the Api 20NE system (bio-Mérieux, Marcy-l'Etoile, France), and antimicrobial susceptibility testing was performed and interpreted as recommended by the Clinical and Laboratory Standards Institute (formerly NCCLS) (*6*).

The antibiogram, which was performed by a disk diffusion method, showed AX476 to be highly resistant to ceftazidime, aminoglycosides, sulfonamides, trimethoprim, and ciprofloxacin but fully susceptible to tetracyclines, piperacillin/tazobactam, ticarcillin/clavulanic acid, and imipenem. Because of an unusual synergy between ticarcillin and ticarcillin/clavulanic acid (Figure), we compared the inhibition zones of third-generation cephalosporin disks with and without clavulanic acid (BioRad, Marnes-la-Coquette, France). The zones were 7 mm for ceftazidime and 19 mm for ceftazidime plus clavulanic acid (Figure), which strongly indicated production of an ESBL. Isoelectric focusing showed that AX476 produced a β-lactamase with an isoelectric point of 7.4. A large plasmid of ≈200 kb (pJDB1) was easily transferred by conjugation to *Escherichia coli* K-12 C600. The transconjugants, *E. coli* (pJDB1) sorbitol-fermenting, which were selected on MacConkey agar that contained 4 μg/mL of ceftazidime, were resistant to sulfonamides and trimethoprim, had reduced susceptibility to aminoglycosides, and harbored a β-lactamase with an isoelectric point of 7.4. The resistance phenotype of the isolate and the value of the isoelectric point of the enzyme suggested the production of the ESBL VEB-1 (*7*).

The MICs for β-lactams for AX476 and its transconjugant, determined by Mueller-Hinton broth dilution method, are shown in Table 1. By using bla_{VEB-1}-specific

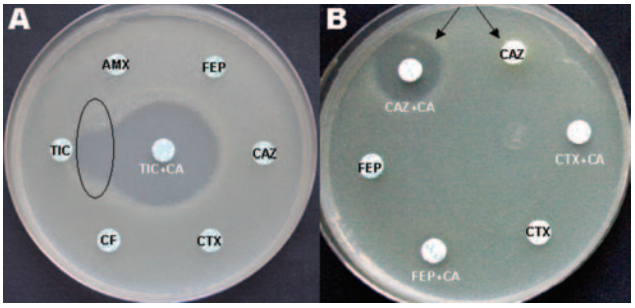

Figure. A) Oval indicates area of synergy between ticarcillin (TIC) and TIC plus clavulanic acid (CA). B) Arrows point to inhibition zone around third-generation disks with and without CA. AMX, amoxicillin; FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime; CF, cefalotin.

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Table 1. Beta-lactam MICs (μg/mL)

β-lactams*	*Achromobacter xylosoxidans* AX476	*Escherichia coli* K-12 C600 (pJDB1)†	*E. coli* K-12 C600‡
Amoxicillin	1,024	16	16
Amoxicillin + CA	32	2	8
Ticarcillin	256	64	8
Ticarcillin + CA	8	2	8
Cefotaxime	>512	0.06	0.03
Cefotaxime + CA	256	0.03	0.03
Ceftazidime	512	2	0.125
Ceftazidime + CA	16	0.125	0.125
Aztreonam	>512	4	0.125
Aztreonal + CA	256	0.125	0.125
Cefepime	512	0.06	0.03
Cefepime + CA	128	0.03	0.03

*CA, clavulanic acid, used at 2 μg/mL.
†Transconjugant.
‡Reference strain.

primers, a positive PCR result was obtained on total DNA from AX476 and the transconjugants. All genetic analyses of bla_{VEB-1} published so far have identified either its chromosome (*8*) or its plasmid (*9*) location and mostly its integration within class 1 integrons of variable structure. Integrons are potentially mobile genetic elements that comprise conserved sequences that flank a variable region and may contain inserted antimicrobial drug resistance gene cassettes (*10*). The 5′-conserved segment includes the gene *intI1* that encodes an integrase, the cassette integration site *attI1*, and a promoter responsible for the expression of the genes located downstream within the variable region. The 3′-conserved region contains either a *qacEΔ1* gene that encodes resistance to quaternary ammonium compounds or a combination of 3 genes: *qacEΔ1*, *sulI* (which encodes resistance to sulfonamides), and *orf5* (an open reading frame of unknown function).

To search for the presence of such a class 1 integron in AX476 and its transconjugant, we performed PCR on total DNA of AX476 and *E. coli* (pJDB1) by using the primers L1 and R1 specific for the detection of class 1 integrons (*11*). We obtained a fragment of 2.3 kb in the clinical strain and its transconjugant, which was sequenced on both strands. By using a set of primers, we deduced the structure of this integron (Table 2). Three gene cassettes have been identified. The first, *dhfr* (dihydrofolate reductase), encoded a putative trimethoprim-resistance protein. This *dhfr* was identical to that reported in *Salmonella enterica* serovar Typhi (GenBank accession no. AL513383) and to the *dhfr* gene cassette contained in a class 1 integron from *Klebsiella pneumoniae* not yet published (GenBank accession no. AJ971342). The second cassette, bla_{VEB-1}, encoded the ESBL VEB-1 first described in *E. coli* (*7*). The third and last gene cassette was *aadB*. It encoded an aminoglycoside adenyltransferase that conferred resistance to kanamycin, gentamicin, and tobramycin and was identical to other sequenced *aadB* gene cassettes located on integrons containing bla_{VEB-1} gene (*7*,*8*). VEB-1 has been detected in *Enterobacteriaceae* and *P. aeruginosa* isolates from Southeast Asia (*9*) but never in *A. xylosoxidans*. In France, VEB-1–producing isolates of *Acinetobacter baumannii* have been involved in several outbreaks of nosocomial infection in intensive care units (*12*,*13*); however, we have not yet detected a VEB-1–producing isolate in our hospital.

Conclusions

This finding of a VEB-1–producing *A. xylosoxidans* from a CF patient enhances the scant information available to laboratorians and clinicians about ESBL production by isolates from CF patients. A very recent study reports 3 ESBL-positive isolates of *P. aeruginosa* from CF patients in New Delhi, but the ESBL has not been characterized (*14*). Resistance to expanded-spectrum cephalosporins mediated by ESBLs has never been described in *A. xylosoxidans*. The detection of the ESBL production was difficult in AX476; therefore, the frequency of *A. xylosoxidans*

Table 2. Primers used for PCRs

Amplified DNA	Primer	Oligonucleotide sequence (5′→3′)	GenBank accession no.
Variable region of class 1 integrons	L1	GGCATCCAAGCAGCAAGC	U49101
	R1	AAGCAGACTTGACCTGAT	U49101
intI1	Int-IN	TGTCGTTTTCAGAAGACGG	U49101
	IntA-R	ATCATCGTCGTAGAGACG	U49101
	IntB-F	GTCAAGGTTCTGGACCAG	U49101
bla_{VEB-1}	VEB-R	GACTCTGCAACAAATACGC	AF010416
	VEB-outF	CAGCAGCCACTAATGATG	AF010416
	VEB-F	CCAGATAGGAGTACAGAC	AF010416
3′CS region	Qac-F	TCGCAATAGTTGGCGAAG	U49101
	Sul-F	GACGGTGTTCGGCATTCT	U49101
	Sul-R	TGAAGGTTCGACAGCACG	U49101
	Orf5-R	GATTTCGAGTTCTAGGCG	U49101
	Orf5-F	GGTGATATCGACGAGGTT	U49101

isolates that produce an ESBL might be underestimated. We recommend the use of BioRad combination disks, especially for isolates that are highly resistant to ceftazidime and susceptible to piperacillin or when synergy exists between ticarcillin and ticarcillin plus clavulanic acid.

The origin of the strain remains unclear. Because *A. xylosoxidans* is widely encountered in the environment, acquisition of AX476 by our patient may have resulted from poor adherence to handwashing, contamination of respiratory therapy equipment (nebulizer), or contaminated water. We can exclude nosocomial acquisition because our patient had never been hospitalized.

The location of bla_{VEB-1} on an easily transferable plasmid might represent a clinical threat if spread among other species widely encountered among CF patients, especially *P. aeruginosa*. Such a transfer would create serious therapeutic problems. Therefore, to prevent person-to-person transmission, our patient visits the physician on different days than the other CF patients. If he needs to be hospitalized, our patient may not share a room with immunocompromised patients or with other CF patients anywhere in the hospital, which is the recommendation for patients with other multidrug-resistant pathogens (*15*). In conclusion, this first finding of a VEB-1–producing *A. xylosoxidans* from a CF patient emphasizes the need to study the mechanism(s) of resistance to ceftazidime among a wide collection of isolates originated from different centers. The sequence of the class 1 integron reported in this paper has been assigned GenBank accession no. DQ393569.

Dr Neuwirth is head of the bacteriology department of the University Hospital and associate professor at the Faculty of Medicine in Dijon, France. She studies the mechanisms of resistance to β-lactam antimicrobial agents among gram-negative bacilli.

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Molecular Characterization of Tickborne Relapsing Fever *Borrelia*, Israel

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Blood samples from 18 tickborne relapsing fever (TBRF) patients and *Ornithodoros tholozani* specimens were tested with a *Borrelia fla*B-PCR. Results were positive for all patients and 2%–40% of ticks. A 7–amino acid gap characterized all 9 sequenced flagellin gene amplicons. By phylogenetic analysis, Israel TBRF *Borrelia* sequences clustered separately from American and African groups.

Tickborne relapsing fever (TBRF) is caused by *Borrelia* species and is transmitted to humans by *Ornithodoros* soft ticks. Worldwide, a dozen *Borrelia* species are known to cause this disease (*1*). In Israel, TBRF is considered to be caused by *Borrelia persica* and transmitted by the cave tick *Ornithodoros tholozani* (*1*). This tick and TBRF are distributed through Central Asia (*2*) and the Middle East (*1*). Other *Borrelia* species that cause TBRF have been described in Iran (*3*), but their precise range of distribution is not known.

In Israel, from 1980 through 2002, 184 cases of TBRF were reported among the civilian population (8 cases/year), and 88% of the case-patients were infected in caves (*4*). Among military personnel, TBRF incidence averages 6.4 cases/100,000 persons (*5*). In Jordan, an average of 72 civilian cases per year was reported from 1959 through 1969 (*6*). In Iran, an average of 100 cases per year has been recently reported (*7*).

TBRF in Israel was first reported by Nicholson (*8*) at the time World War I. Detailed clinical and epidemiologic features of the disease are well described in this article, particularly the transmission by ticks (*8*). However, Nicholson incorrectly attributed the disease to the soft tick *Argas persicus*. In 1937, Adler et al. clearly identified *O. papillipes* (*tholozani*) as the vector of the disease (*9*) and characterized the causative agent as *Spirochaeta persica* (*10*).

Although American (*11*) and African TBRF *Borrelia* (*12*) are now better characterized, no definitive molecular characterization of *Borrelia* species responsible for relapsing fever has been achieved in Israel. The aim of this study is to provide initial molecular characterization of the etiologic agent of TBRF in Israel from both ticks and human samples and to compare it with previously described agents of TBRF in other parts of the world.

The Study

We designed a genus-specific set of primers (BOR1: 5′ TAA TAC GTC AGC CAT AAA TGC 3′and BOR2: 5′ GCT CTT TGA TCA GTT ATC ATT C 3′) that targeted the *Borrelia fla*B flagellin gene (*13*). Each PCR mixture (25 μL) contained 3 μL of target DNA and was subjected to 1 min at 95°C, followed by 40 cycles of 56°C for 30 sec, 72°C for 30 sec, 94°C for 30 sec, and 5 min at 72°C for final elongation. DNA of *B. duttonii* and *B. burgdorferi* sensu stricto (strain B31) was used as controls. DNA of blood and ticks was extracted with the DNA easy tissue kit (Qiagen, Hilden, Germany). Tick samples were collected by using CO_2 traps in caves and were identified as *Ornithodoros tholozani* (Figure 1) by the Entomology Laboratory (Ministry of Health, Jerusalem). The tick specimens collected were tested either individually or as pools. Of 184 tick specimens collected from 5 different areas (Table 1), 94 were tested by BOR1-BOR2 PCR. One pool of 5, a pool of 4, and 6 individual specimens were positive; all produced a unique band 750 bp in length. The percentage of tick infection was variable, ranging from <2% in Ma'ale-Adumim to 40% in the Be'er Sheva region.

For patients, the TBRF diagnosis was established as previously reported (*5*). Eighteen samples of human blood were sent to the Parasitology Reference Center (Ministry of Health, Jerusalem); the samples corresponded to 15 confirmed cases (positive blood smear) and 3 associated cases of TBRF (negative blood smear). On receipt at the laboratory, fresh human blood samples were examined by darkfield microscopy for viable *Borrelia* and, if detected, 200 μL of blood was injected into 10-mL vials of BSK-H medium (*14*) and into 10-week-old ICR mice by the intraperitoneal route. In 4 patients, blood examined by

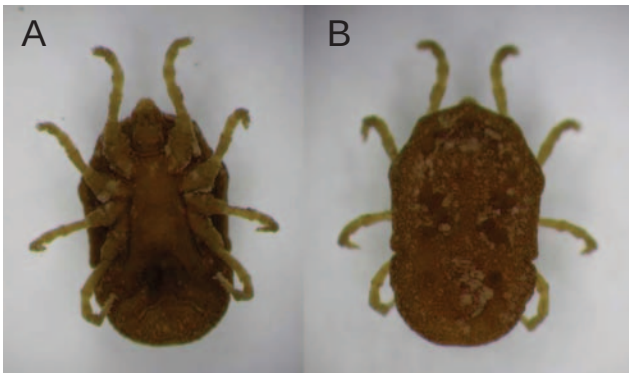

Figure 1. *Ornithodoros tholozani* ventral (A) and dorsal (B) views.

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Table 1. Percentage of tick infestation by PCR at several locations

Region/location	No. ticks collected	No. tested	Pools/individuals	PCR result	% Infestation
Jerusalem outskirts					
Ma'ale Adumim	51	51	9 individuals	–	<1.9
			5 pools of 5	–	
			3 pools of 4	–	
			5 individuals	–	
Jericho					
Makoh	30	10	Pool of 4	–	6.6
			6 individuals	1+	
Center					
Gimzo	45	15	Pool of 5	+	13–40
			Pool of 3	–	
			Pool of 3	–	
			4 individuals	1+	
Tiberias					
Migdal	8	8	Pool of 4	+	12.5–50
			Pool of 4	–	
Be'er Sheva, Arad Valley					
Hurvat Kasif	50	10	10 individuals	4 positive	40

darkfield microcopy showed 1–5 motile *Borrelia* per slide. In vitro cultivation was unsuccessful. However, *Borrelia* (1–5/field) were detected on day 4 (twice) and day 6 (twice) in the blood of mice injected intraperitoneally with patient blood. Cultivation attempts from positive mice blood were also unsuccessful. In contrast, all the samples were found positive by BOR1-BOR2 PCR, showing a unique band of 750 bp (data not shown).

PCR products were cloned in T7 plasmid by pGEM-T Easy vector SystemII (Promega, Madison, WI, USA). Plasmids containing inserts were purified and sent for 2-strand sequencing. Direct sequencing of DNA amplified by the BOR1 and BOR2 primers was performed later.

Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.1 (*15*). Among published *fla*B genes of TBRF *Borrelia* strains, only sequences for which a translated protein existed were taken in account. Because of the large number of available sequences for American *Borrelia* associated with TBRF, as well as for *B. duttonii* and *B. recurrentis*, a single sequence representative of each cluster was chosen for taxonomic analyses.

Three PCR amplicons (from 1 tick and 2 human samples) were sequenced after cloning, whereas 6 amplicons (from 2 ticks and 4 human samples) were analyzed by direct sequencing. These 9 sequenced samples showed 98%–100% homology between them and could be divided into 3 groups. The same DNA sequence was found in tick TG52 and in blood from 2 patients, HumanBlood2 and HumanBlood4. These 3 sequences had an additional triplet at the position 627. The second group of sequences, which consisted of tick samples TGd1 and CBkc7 and blood samples C1025B, FL1, and HumanBlood3, were identical, with only 3 minor substitutions between them. The third group consisted of the HumanBlood1 sample.

All the translated sequenced amplicons showed a very specific signature by the presence of a 7–amino acid (aa) gap at position 216 (see online Appendix Figure, available from http://www.cdc.gov/ncidod/EID/vol12no11/06-0715-appG.htm) when compared with previously described TBRF *Borrelia fla*B genes. In addition, the local TBRF *Borrelia* sequences could be grouped into 3 subtypes, according to variation at 7-aa positions (Table 2).

Comparison with published *fla*B protein sequences of TBRF *Borrelia* showed 88%–90% homology with *B. duttonii* and *B. recurrentis*, 85%–90% with *B. crocidurae*, 86%–88% with *B. turicatae*, 87%–89% with *B. hermsii*, and 85%–88% with *B. parkeri*. The sequences of the Israel TBRF *Borrelia* isolated from different samples clearly clustered in a separate group from the American and the African TBRF species (Figure 2).

Table 2. Variable amino acid (aa) positions and type definition of the *flaB* gene for the 9 samples sequenced for the Israeli tickborne relapsing fever *Borrelia persica*

	Position*							
Type	86	105	134	195	216	228	231	No. strains
I	V	A	R	A	-	I	I	5
II	V	A	R	S	A	I	I	3
III	I	S	H	A	-	T	V	1

*Numbering according the aa sequence of *B. hermsii* gi|1311448|.

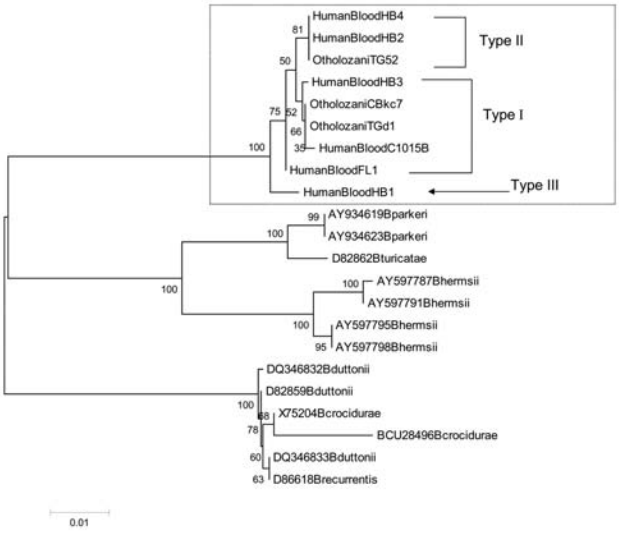

Figure 2. Phylogenetic tree based on *fla*B nucleotide sequences. The tree was constructed by the neighbor-joining method in a pairwise deletion procedure. Distances were calculated according to the Jukes and Cantor method. Numbers at nodes correspond to the percentage confidence level in a bootstrap test performed on 1,000 replicates. The scale bar corresponds to a 0.01 distance. The GenBank accession numbers for nucleotide sequences of *Borrelia persica fla*B shown here are as follows: HumanBloodFL1 (DQ673617), HumanBloodC1015B (DQ679904), OtholozaniCBkc7 (DQ679905), HumanBlood1 (DQ679906), HumanBlood2 (DQ679907), HumanBlood3 (DQ679908), HumanBlood4 (DQ679909), OtholozaniTG52 (DQ679910), and OtholozaniTGd1 (DQ679911).

Conclusions

Our results suggest that infection rates differ according to location, despite the small number of ticks tested and the use of pools. BOR1-BOR2 PCR was more sensitive than blood smear examination (100% vs 83%). An identical DNA sequence was found in both tick and patient samples and thus confirms, at the molecular level, the role of *O. tholozani* as the vector of TBRF in Israel.

A signature (7-aa gap) of the *fla*B flagellin defined the Israeli TBRF sequences as a homologous group different from other TBRF species. Despite the small number of samples studied, a clear polymorphism existed also at the protein level, resulting in 3 local types. This diversity can be explained by the use of direct sequencing of samples rather than through cultivation that reduces the biodiversity of isolates by selecting the most successful in vitro clone.

This study opens a new avenue in TBRF *Borrelia* studies by demonstrating a Middle East cluster in addition to the American and African groups. These results open encouraging perspectives for the better understanding of entomologic, epidemiologic, and bacteriologic aspects of this disease and may contribute to better diagnosis and treatment.

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Fatal Avian Influenza A H5N1 in a Dog

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Avian influenza H5N1 virus is known to cross the species barrier and infect humans and felines. We report a fatal H5N1 infection in a dog following ingestion of an H5N1-infected duck during an outbreak in Thailand in 2004. With new reports of H5N1 virus continuing across Asia, Europe, and Africa, this finding highlights the need for monitoring of domestic animals during outbreaks.

Highly pathogenic avian influenza (HPAI) H5N1 has spread across Asia, Europe, and Africa. Not limited to poultry, the virus has also been shown to cross the species barrier infecting humans (*1*) and felines, including domestic cats (*2*) and tigers (*3–5*). Both cats and tigers were reported as becoming infected after eating poultry carcasses harboring HPAI. Here, we report a case of HPAI H5N1 infection in a domestic dog following ingestion of the carcass of an infected duck.

The Study

In October 2004, the carcass of an a ≈1-year-old dog from Suphanburi Province, Thailand, was submitted for necropsy at the Faculty of Veterinary Medicine, Kasetsart University, in Nakorn Pathom, Thailand. The dog's owner stated that the dog had eaten duck carcasses from an area with reported HPAI H5N1 infections in ducks. Approximately 5 days after ingesting the carcasses, the dog developed high fever, panting, and lethargy and died on the following day. Within 4 hours of its discovery, the dog carcass was sent to the laboratory.

Necropsy findings included bloody nasal discharge; severe pulmonary congestion and edema (Figure 1A); and congestion of the spleen, kidney, and liver. Brain, lung, trachea, heart, duodenum, jejunum, ileum, liver, spleen, kidney, pancreas, and urine specimens were obtained separately and processed for virus isolation by injection into 10-day-old embryonated chicken eggs. Forty-eight hours later, allantoic fluids harvested from dead embryos that had been injected with supernatants of ground brain, trachea, lung, intestine, liver, and kidney were tested with the hemagglutination and hemagglutination-inhibition tests. Influenza virus was isolated from lung, liver, kidney, and urine specimens, and the viral subtype was determined to be H5N1 by reverse transcription (RT)–PCR (*6*). The 4 tissues that showed virus were also processed for histopathologic and immunohistochemical analysis. Immunohistochemical tests were performed on paraffin-embedded tissues by using a mouse monoclonal antibody anti-nucleoprotein of influenza A H5N1 (B.V. European Veterinary Laboratory, Woerden, the Netherlands) as a primary antibody and a polyclonal goat antimouse immunoglobulin G tagged with peroxidase as a secondary antibody (DAKO A/S, Glostrup, Denmark). Diaminobenzidine was used as a substrate. Positive lung tissue from the dog that was incubated with phosphate-buffered saline instead of the mouse monoclonal antibody antinucleoprotein of influenza A H5N1, and tissue from the liver and lung of a cat killed by a car served as negative control (*2*).

Histopathologic examination of the lung showed severe pulmonary edema and interstitial pneumonia with inflammatory cell infiltration. Hemolysis with brownish black particles was found in the pulmonary parenchyma (Figure 1B), and the liver showed focal necrosis (Figure 1C). The kidneys showed mild nephritis with tubular degeneration. No microscopic lesions were found in any other organs. On immunohistochemical analysis, positive sites were found in alveolar cells, hepatic cells (Figure 1D), renal

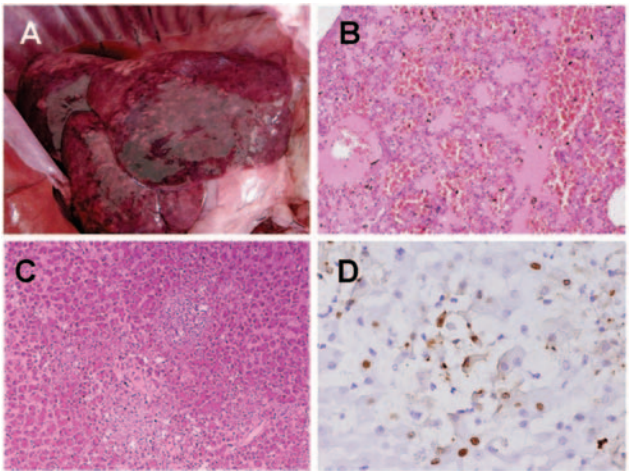

Figure 1. Gross and microscopic lesions from dog infected with highly pathogenic avian influenza (HPAI) H5N1. A) Severe congestion and edema in the lung. B) Lung histopathologic results showing severe pulmonary edema and hemorrhage with black-brown particles (hemosiderin) (magnification ×100). C) Liver histopathologic changes showing necrotic foci (pale area) (magnification ×100). D) Immunohistochemical results: the nucleoprotein of the virus is detected in nuclei of hepatocytes with brown granule (magnification ×200).

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tubular epithelium, and glomerulus; none of the remaining organs were positive for H5N1.

H5N1 viruses were isolated from the dog's lung tissue and designated A/Dog/Thailand/KU-08/04. Genetic analysis was used to characterize the dog's virus (KU-08), and the sequences were deposited at GenBank under accession number DQ530170–7. Sequencing and phylogenetic analysis of the hemagglutinin (HA) and neuraminadase (NA) genes of the dog's virus showed that they were similar to those of H5N1 viruses isolated from tigers, chickens, ducks, and humans infected in Thailand during the same time that the dog was infected (Figure 2A and B). In addition, analysis of 6 other genes from KU-08 showed similar results (data not shown). Phylogenetic analysis clearly indicated that all the Thailand isolates were clustered with the Vietnam lineage, which groups separately from the Indonesia lineages and China (Qinghai), Europe, and Africa lineage. Genetic comparisons of the 8 genes analyzed from KU-08 to those of viruses isolated in Thailand from chickens (Jan 04, Jul 04, Oct 05), tigers (Jan 04, Oct 04), humans (Jan 04, Dec 05), cats (Jan 04), and geese (1996, Jun 05) are shown in the Table. The analysis showed that KU-08 was more closely related to the tiger isolate (CU-T3) obtained in Oct 2004, with higher percentages of nucleotide identity (100% identity for 5 genes: H5, N1, matrix [M], nonstructural [NS], polymerase basic protein 1 [PB1]) compared to any of the Thailand isolates obtained from early 2004 and late 2005.

This finding indicates that the dog's H5N1 infection resulted from the virus circulating during the second wave of H5N1 outbreaks that occurred in Thailand during mid-2004. The HA gene of KU-08 contained multiple basic amino acid insertions at the HA cleavage site (SPQRERRRKKRR), similar to those found at the HA cleavage site for other viruses characterized from Thailand during this time. However, the isolates from the third wave of AI outbreaks that occurred in Thailand in 2005 contained 1 basic amino acid (aa) change at the HA cleavage site (SPQREKRRKKRR) (*7*). Moreover, the viruses isolated from China (A/Bar-headed goose/Qinghai/65/05, A/Black-headed gull/Qinghai/1/05) (*8*), Iran (A/Cygnus cygnus/Iran/754/06), Russia (A/Cygnusolor/Astrakhan/Ast05–2-9/05), and Nigeria (A/chicken/Nigeria/641/06) displayed a different amino acid at the HA cleavage site (SPQGERRRKKRR). The receptor-binding site of the dog isolate still exhibited avian characteristics in that it contained glutamine (Q) and glycine (G) at positions 222 and 224 of the HA gene (Q222–G224). The NA gene of KU-08 also had 20 aa deletions at positions 49–68 and contained histidine (H) amino acid at position 274, indicating the absence of an oseltamivir-resistant residue. The NS gene of the KU-08 isolate contained a 5-aa deletion at positions 79–83, and the M gene of the KU-08 isolate displayed an amantadine-resistant amino acid (N31; asparagine). In summary, the viruses from the dog were similar to the H5N1 viruses isolated in Thailand in 2004 and to the Vietnam lineage which had been identified as genotype Z (*9*). A single amino acid substitution at position 627 of the PB2 gene (glutamic acid [E] to lysine [K]) was observed in KU-08 and had previously been observed in human, tiger, and cat isolates from Thailand as well as the viruses from China (Qinghai). The presence of lysine (K) may relate to more efficiency of viral replication in mammal species (*10*). On the other hand, in pigeon isolates from Thailand (KU-03), the PB2–627 aa residue remained unchanged (E; glutamic acid).

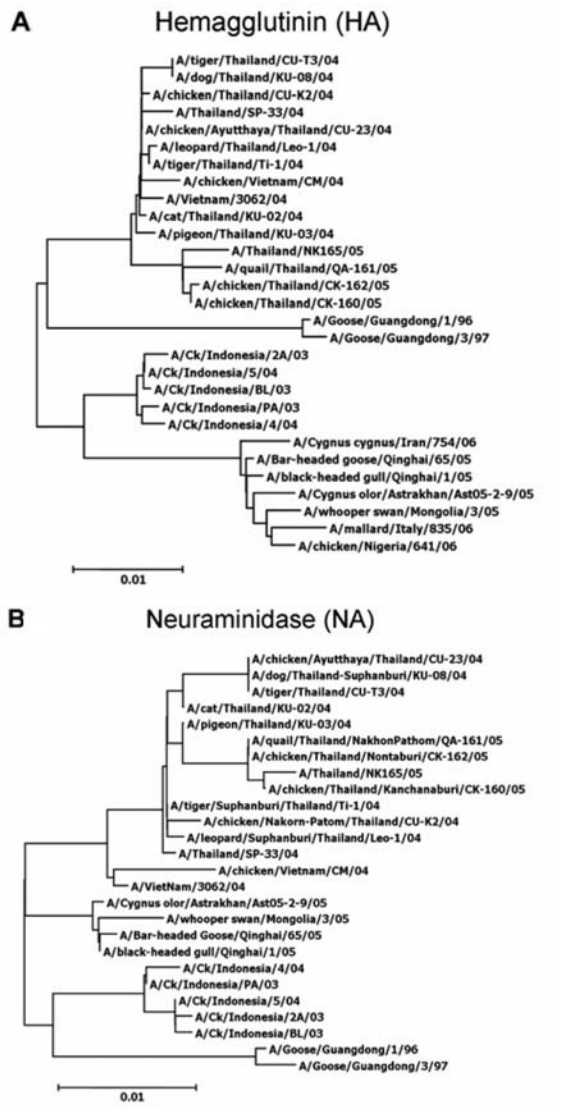

Figure 2. Phylogenetic analysis of the hemagglutinin (A) and neuraminidase (B) gene sequences of the H5N1 influenza virus isolated from a dog's lung (KU-08), compared with other HA and NA sequences stored in GenBank. For a larger reproduction of the phylogenetic analysis, see online figure available from http://www.cdc.gov/ncidod/EID/vol12no11/06-0542-G2.htm

Table. Genetic comparison of 8 gene segments of the H5N1 influenza virus isolated from the dog's lung (KU-08) to those of H5N1 isolates from Thailand*

		% nucleotide identity									
KU-08: dog (Oct 04)†	Region of comparison	GD-1: goose (1996)‡	CU-K2: chicken (Jan 04)§	SP-33: human (Jan 04)¶	Ti-1: tiger (Jan 04)#	KU-02: cat (Jan 04)**	CU-23: chicken (Jul 04)††	CU-T3: tiger (Oct 04)‡‡	CK-160: chicken (Oct 05)§§	NK-165: human (Dec 05)¶¶	QH-65: goose (Jun 05)##
HA	46–1623	96.0	97.1	99.0	99.6	99.6	99.6	100.0	99.2	98.9	96.8
NA	28–1296	91.6	99.3	99.3	99.5	99.5	99.9	100.0	98.5	98.8	97.2
M	1–801	96.1	99.5	99.6	99.4	99.9	99.9	100.0	99.4	99.3	98.1
NS	37–789	68.5	99.0	99.2	99.3	99.5	99.3	100.0	99.1	99.0	96.4
NP	58–1428	92.6	99.7	99.7	99.6	99.6	99.8	99.9	99.1	99.1	98.1
PA	28–2118	93.5	99.4	99.3	99.5	99.4	99.5	99.7	99.2	98.8	97.7
PB1	67–2229	93.4	99.6	99.8	99.8	99.8	99.9	100.0	99.1	98.8	97.9
PB2	88–2220	94.0	99.4	99.3	99.4	99.7	99.8	99.8	98.9	98.9	97.5

*HA, hemagluttinin; NA, neuraminadase; M, matrix; NS, nonstructural; NP,nucleoprotein; PA, polymerase A; PB, polymerase basic protein.
†A/dog/Thailand-Suphanburi/KU-08/04 (DQ530170–7).
‡ A/Goose/Guangdong/1/96 (AF144300–7).
§A/chicken/Nakorn-Patom/Thailand/CU-K2/04 (AY590567- 8, AY590578–82, AY551934).
¶A/Thailand/SP-33/04 (AY555152–3, AY627893–8).
#A/tiger/Suphanburi/Thailand/Ti-1/04 (AY646167–74).
**A/cat/Thailand/KU-02/04 (DQ236077–84).
††A/chicken/Ayutthaya/Thailand/CU-23/04 (AY770991- AY7709918).
‡‡A/tiger/Thailand/CU-T3/04 (AY907672–3, AY842935–6, AY972547–50).
§§A/chicken/Thailand/Kanchanaburi/CK-160/05 (DQ334757–64).
¶¶A/Thailand/NK165/05 (DQ372591–8).
##A/Bar-headed Goose/Qinghai/65/05 (DQ095622, DQ095642, DQ095662, DQ095682, DQ095702, DQ095722, DQ095742, DQ095762).

This study is the first report of H5N1-related systemic disease in a domestic dog infected during the second wave of outbreaks in Thailand that occurred during October 2004. The most plausible route of the dog's infection was ingestion of infected duck carcasses. Previous studies have shown that avian viruses preferentially recognize α-2,3 linkage (SAα2,3Gal) and bind to type II alveolar cells, which are abundant in the lower respiratory tract of mammals (*11*,*12*); these findings support our observations of severe pneumonia with lung edema in the infected dog (Figure 1). Characterization of the H5N1 isolates from the dog showed identical properties to the H5N1 isolates from the Thai epidemic. Moreover, genetic comparison indicated that the dog isolate was similar to the H5N1 viruses recovered from a tiger (CU-T3) in Thailand during the mid-2004 epidemic.

Conclusion

Our results demonstrate that, as has previously been shown for cats, dogs are at risk for H5N1 infection. Despite the low probability of H5N1 infection in domestic animals, the possibility of humans acquiring H5N1 infection from direct contact with infected cats and dogs warrants concern and highlights the need for monitoring domestic animals during H5N1 outbreaks in the future.

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Reemergence of Dengue Virus Type 4, French Antilles and French Guiana, 2004–2005

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After 10 years of absence, dengue virus type 4 (DENV-4) has recently reemerged in Martinique, Guadeloupe, and French Guiana. Phylogenetic analyses of strains isolated from 2004 to 2005 showed that they belong to DENV-4 genotype II, but to a different cluster than strains isolated from 1993 to 1995.

Dengue is a viral disease transmitted by mosquitoes. It is caused by any of the 4 viral serotypes of dengue virus (DENV), designated DENV-1, DENV-2, DENV-3, and DENV-4. DENV belongs to the genus *Flavivirus* and family *Flaviviridae*. Flaviviruses are enveloped, single-stranded, positive-sense RNA viruses. The genomic RNA is ≈11 kb and has 10 genes coding 3 structural proteins (capsid [C], envelope [E], and membrane [M]), and 7 nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5).

Dengue is the predominant arthropodborne viral disease affecting humans. DENV causes a wide range of symptoms from inapparent or mild disease (dengue fever) to severe forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The disease is now endemic in >100 countries and threatens >2.5 billion people. It currently occurs in tropical areas and affects ≤100 million persons each year (*1*). Of these persons, 500,000 have DHF and ≈25,000, mainly children, die. The World Health Organization has estimated a 30-fold incidence increase in dengue the past 50 years (*2*).

DENV is transmitted to humans by *Stegomyia aegypti* (formerly *Aedes aegypti*) mosquitoes. Before the 1980s, epidemic dengue was rare in the Americas because *St. aegypti* had been eradicated from most Central and South American countries. In the 1990s, *St. aegypti* had almost completely reinvaded the regions in which it was found before its eradication. Within the past 30 years, the increase in the worldwide transportation network, as well as uncontrolled population growth and urbanization, has led to larger and more frequent DENV epidemics and more cases of DHF/DSS (*3*).

During the past 20 years, the 4 DENV serotypes have been isolated in the French departments of the Americas: Guadeloupe, Martinique, and French Guiana. Martinique and Guadeloupe are 2 Caribbean islands located in the Lesser Antilles and represent the French West Indies, and French Guiana is located southeast of the French West Indies in northern South America between Suriname and Brazil. DENV is endemic in French Guiana. Dengue epidemics occurred in this country at 4–6-year intervals from the 1960s to the early 1990s (*4*). The first DHF cases were associated with a DENV-2 outbreak in 1991–1992 (*5*). DENV-4 was sporadically isolated between 1993 and 1995. Cocirculation of DENV-1 and DENV-2 caused outbreaks between 1996 and 1998, followed by 2 consecutive DENV-3 epidemics in 2001–2002 and 2004–2005. Martinique had 3 dengue epidemics during the past decade in a setting of sporadic transmission and seasonal peaks from July to December. These epidemics were associated with DENV-2 and DENV-4 in 1995, DENV-1 in 1997, and DENV-3 in 2001 (*6*). The epidemiology of DENV in Guadeloupe is similar to that in Martinique, although no data are available about previous dengue epidemics.

The Study

Our study reports the evolution of DENV-4 in French Guiana and the French Caribbean islands since its last detection and circulation from 1993 to 1995. We studied 8 DENV-4 strains isolated from human sera from French Guiana in 1993 and 1995 (n = 6) and in 2004 and 2005 (n = 2) (Table). We also tested 3 human serum specimens from Martinique (n = 2) and Guadeloupe (n = 1) that were positive for DENV-4 during dengue surveillance in the fourth quarter of 2004.

DENV-4 infection was confirmed by using virus isolation on AP 61 cells as previously described (*7*). We then amplified from first-passage RNA a 1,940-bp region of the genome for the E gene and adjacent prM/M and NS1 junctions previously described (*8*) to analyze the phylogenetic relationships of these strains. Each PCR product was cloned by using the TOPO TA Cloning kit (Invitrogen, Paisley, UK). For each isolate, 3 clones were sequenced by Genome Express (Meylan, France).

We compared our 11 sequences to the 87 DENV-4 sequences available in the GenBank database. Sequences were aligned by using MacVector version 7.2 software (Oxford Molecular Ltd., Oxford, UK). Percentage of nucleotide identity between sequences was determined for all observed differences (including insertions and deletions)

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Table. Dengue virus type 4 strains used for the phylogenetic reconstruction

Country of origin	Strain	Year of isolation	GenBank accession no.
French Guiana	FGU-Dec-1993	1993	DQ390322
	FGU-Mar-1994, -Jun-1994, Sep-1994	1994	DQ390325, DQ390327, DQ390329
	FGU-Feb-1995, -Jun-1995	1995	DQ390324, DQ390326
	FGU-Oct-2004	2004	DQ390328
	FGU-Fev-2005	2005	DQ390323
Guadeloupe	GUA-FWI-Dec-2004	2004	DQ390320
Martinique	MAR-FWI-Aug-2004, Dec-2004	2004	DQ390319, DQ390321
Bahamas	BAH 1998 A, B, C	1998	AY152364–66
Barbados	BDS 1993 A, B	1993	AY152375–76
	BDS 1999	1999	AY152368
Costa Rica	CRA 1996	1996	AY152104
Dominica	DOM 1981	1981	AY152360
Ecuador	ECD 1994	1994	AY152292
El Salvador	ELS 1993	1993	AY152300
Honduras	HON 1991	1991	AY152379
Jamaica	JAM 1981	1981	AY152389
	JAM 1983	1983	AY152384
Martinique	MAR-FWI 1995	1995	AY152100
Mexico	MEX 1991	1991	AY152378
	MEX 1995	1995	AY152304
Monserrat	MON 1994 A, B, C, D	1994	AY152369–71
Puerto Rico	PR 1982 M03, M05	1982	AY152336, AY152344
	PR 1985 M32, M33	1985	AY152856–57
	PR 1986 115	1986	AY152224
	PR 1987 67, 73	1987	AY152236, AY152268
	PR 1990 96	1990	AY152855
	PR 1992 24, 35, 36	1992	AY152112, AY152188, AY152208
	PR 1994 81, 83	1994	AY152144, AY15148
	PR 1998 13, 17	1998	AY152056, AY152068
Suriname	SUR 1982 A, B, C, D	1982	AY152385–88
	SUR 1994 A, B, C	1994	AY152372–74
Trinidad	TRI 1982 A, B	1982	AY152382–83
	TRI 1984 A, B	1984	AY152380–81
	TRI 1994	1994	AY152377
	TRI 1999	1999	AY152367
Venezuela	VEN 1995	1995	AY152092

and was calculated for the 1,473 nt available for all 98 sequences analyzed. Sequence comparison of strains from Martinique, Guadeloupe, and French Guiana with those reported from Asia showed that nucleotide divergence is 8%–12%. The level of nucleotide divergence was lower for sequences from the Caribbean area (maximum nucleotide divergence 2.8%). All 11 newly obtained sequences were different from each other. These sequences exhibited a 0.06%–0.47% nucleotide divergence (strains isolated in 1993 and 1995) and a 0.06%–1.22% nucleotide divergence (strains isolated in 2004 and 2005) among themselves. These strains had amino acid changes at position 351 in the E gene (from isoleucine to valine) and position 52 in the NS1 gene (from lysine to arginine), which supports their inclusion in the modern Caribbean basin clade (*8*).

We conducted phylogenetic analyses on nucleotide sequences by using distance and parsimony methods in PAUP* version 4.0b8 to increase the reliability of derived tree topologies (*9*). We also used the neighbor-joining distance matrix algorithm with the Kimura 2 parameter (Figure) and the heuristic algorithm for the maximum parsimony analysis (data not shown). Robustness of resulting topologies was examined by using bootstrap analyses (*10*). Both neighbor-joining and parsimony algorithms underwent 1,000 iterations.

The phylogenetic tree constructed with the neighbor-joining method on nucleotide sequences restricted to all American DENV-4 isolates available in GenBank (Table) is shown in the Figure. Our 11 strains segregate into 2 main clades that were identified in the 2 phylogenetic analyses from consistent topologic associations and high bootstrap values. The strains isolated from French Guiana in 1993 and 1994 clustered with strains from Suriname and Costa Rica, which were isolated at the same time, as well as with a previously reported strain from Martinique isolated in 1995. Our 5 strains isolated in 2004 and 2005 are related to a group of DENV-4 genotype II sequences

isolated in the Bahamas in 1998. This finding suggests that strains circulating in French departments of the Americas in 2004 and 2005 probably evolved from strains detected in the Caribbean islands during the late 1990s. Our observations confirm that DENV-4 lineages in the Caribbean and nearby regions are grouped temporally rather than by the geographic origin of the isolates, as has been previously suggested (*8*).

Conclusions

The DEN-4 serotype was first reported in the Americas in 1981. At that time, isolates collected in the Americas were similar to strains collected in Southeast Asia. This lineage was designated DENV-4 genotype II (*11*). Since it was first detected in Dominica and the French islands of Saint Barthelemy and Saint Martin (*12*), this genotype has spread rapidly throughout the Caribbean and Latin America and caused DF and sporadic cases of DHF/DSS (*3*).

Figure. Phylogenetic tree of American dengue virus type 4 (DENV-4) strains generated by using the neighbor-joining algorithm with Kimura 2 parameter distance. Numbers above branches refer to bootstrap values generated by using distance, and numbers under nodes refer to bootstrap values generated by using parsimony. Names of isolates refer to country and are listed in the Table. Accession no. M14931 refers to the complete genome of DENV-4. A and B refer to the 1981 introduction group and to the modern Caribbean clade, respectively, and B1-B4 correspond to 4 different lineages within group B, as previously reported by Foster et al. (*8*).

DENV-4 genotype II never stopped circulating in the Caribbean region, despite its being periodically locally eliminated (*8*). This genotype reappeared in French departments of the Americas in 2004 after the introduction of a strain circulating in neighboring Caribbean islands, rather than by reemergence of a strain detected 10 years ago. Furthermore, this DENV-4 genotype was detected in French Guiana (October 2004) a few weeks after it was detected in Martinique (August 2004), which suggests that the strain came from the Antilles (Table). A similar phenomenon had already been observed in 1999 for the DENV-3 genotype (J. Morvan, unpub. data). This model of viral spread may be caused by cultural and economic ties between French departments, which allows gene flow through the Caribbean islands and South America as persons with viremia move through the region. This reflects the general pattern of dengue evolution in the Americas (*13*,*14*).

Reintroduction of DENV-4 in the French Antilles and French Guiana in 2004 after a decade of absence highlights the risk for subsequent epidemics in persons with no immunity to dengue. As expected, the French Antilles had a major DENV-4 epidemic with cocirculation of DENV-2 from July 2005 to January 2006 (*15*). In contrast, only sporadic cases of DENV-4 infection have been observed in French Guiana since the beginning of 2005. This indicates that other factors such as mosquito density or human susceptibility, in addition to immune status of the host or silent transmission of the virus, can modulate the risk for dengue epidemics.

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Genotype III Saint Louis Encephalitis Virus Outbreak, Argentina, 2005

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Twenty-six years after it was last detected, Saint Louis encephalitis virus (SLEV) genotype III reemerged in 2005 in Córdoba, Argentina, where it caused an outbreak. Two genotype III SLEV strains were isolated from *Culex quinquefasciatus*. A 71.43% prevalence for neutralizing antibodies was found in domestic fowl in the homestead of a patient with encephalitis.

Saint Louis encephalitis virus (SLEV; genus *Flavivirus*, family *Flaviviridae*) emerged in Córdoba Province, Argentina, in 2002. A single case of human encephalitis occurred (*1*). An outbreak of SLEV with 47 laboratory-confirmed cases, 9 fatal, occurred in Córdoba Province in 2005 (*2*). SLEV is widely distributed in the United States and in Central and South America, maintained in transmission cycles involving *Culex* mosquitoes and various birds (*3*). According to serologic data, SLEV is distributed throughout Argentina, including subtropical provinces in the north to the cold temperate province of Rio Negro in the south. Sporadic symptomatic cases of Saint Louis encephalitis (SLE) have been reported since 1964 (*4*). SLEV strains have been isolated from *Culex* mosquitoes, rodents, and febrile humans. Serologic evidence of natural infection has been reported in horses, goats, cattle, and wild and domestic birds (*4*). To investigate the etiology of the human encephalitis outbreak, we sought to detect and characterize a viral agent from mosquitoes and evaluate prevalence of SLEV-neutralizing antibodies in domestic birds in Córdoba.

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The Study

During a human encephalitis outbreak in February 2005, we collected adult mosquitoes and blood-sampled domestic geese and chickens at an urban residence of a patient with confirmed SLE. In 1 night with light traps, we collected 393 mosquitoes: *Aedes aegypti* (2.8%), *Cx. interfor* (13%), *Cx. quinquefasciatus* (84%), and *Ochlerotatus albifasciatus* (0.2%). Seven pools of female mosquitoes were organized and processed as previously described (*5*). A *Flavivirus*-generic reverse transcription (RT)–PCR assay was used to detect flavivirus-infected mosquito pools, and cDNA amplicons were sequenced as previously described (*6*), resulting in 3 SLEV-positive pools of unengorged females: pools 4005 and 4006 of *Cx. quinquefasciatus* and 4002 of *Cx. interfor* (GenBank accession nos. DQ232620, DQ232621, and DQ232619, respectively). An aliquot of 0.1 mL of each positive pool was injected onto a Vero cell monolayer, and 2 SLEV strains, CbaAr-4005 and CbaAr-4006, were isolated from the 2 *Cx. quinquefasciatus* pools. Both strains required 4 blind passages after 6 days of incubation in Vero cells until cytopathic effect was observed on day 6 postinjection. The harvested supernatant and cells of the fourth passage contained 6 $\log_{10}$/mL PFUs. These 2 strains were reisolated from the original mosquito pools with the same technique.

To characterize the isolated SLEV strains, their E genes were sequenced after RT-nested-PCR amplification with primers SLE-841S 5′-GGTTTTGCCGCAATCCTGGNTAYGC-3′, SLE-869S 5′-AGTTGCGCTGGCGATTGGNTGGATG-3′, SLE-2546AS 5′-GAAATACTTGTAGTCACTCRTCCAC-3′, and SLE-2541AS 5′-ACTTGTAGTCACTCTTCCAYTTYTC-3′. The phylogenetic analysis was conducted with MEGA version 3.0 (*7*). Sequences were aligned with 71 other SLEV sequences available in GenBank and 3 other related flaviviruses as outgroups (WNV M12294, JEV M18370, and MVEV AF161266). Isolated viral strains were categorized in genotypes by using the classification proposed by Kramer and Chandler (*8*).

The 3 sequences derived from the positive mosquito pools (4002, 4005, and 4006) were identical except for 8 silent substitutions (among 87 nt analyzed) and were closely homologous to SLEV sequence AF013416, with a high bootstrapping value (999/1,000). Subsequently, the entire E glycoprotein gene was sequenced from the 2 cultured isolates (GenBank accession nos. DQ385451 and DQ385450), and a phylogenetic tree was derived (Figure). The closest related GenBank sequence was AF205490 (bootstrap value 999/1,000), corresponding to the 79V2533 strain of SLEV isolated from a pool of *Culex* mosquitoes collected in Santa Fe Province, Argentina, in 1979. Both strains we isolated, therefore, belong to the genotype III described by Kramer and Chandler (*8*).

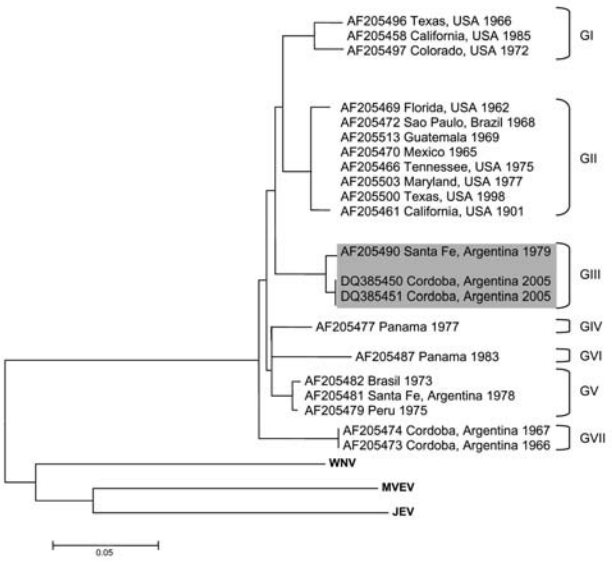

Figure. Consensus tree of the maximum parsimony analyses of Saint Louis encephalitis virus and other related flavivirus E glycoprotein genes. Shading indicates the genotype III to which the new viral strain belongs. West Nile virus (WNV), Japanese encephalitis virus (JEV) and Murray Valley encephalitis virus (MVEV) are used as outgroups. Scale bar indicates number of nucleotide differences

Blood samples (0.2 mL) were taken from the jugular vein (chicks) or brachial vein (hens, geese) with a 27-gauge 3/8-inch needle attached to a 1-mL syringe and added to 0.9 mL sterile phosphate-buffered saline, for an ≈1:10 dilution of serum. Sera were tested for neutralizing antibodies by using the plaque-reduction neutralization test (*9*). Neutralizing antibodies against SLEV were detected in both geese and chickens (Table).

Conclusions

The Córdoba outbreak in 2005 represents the first reported SLE outbreak in Central and South America. Before 2005, the only recorded outbreak of human encephalitis caused by flaviviruses in this region was in 1975 in Brazil, which was attributable to Rocío virus (*10*). The finding of genotype III SLEV strains in Córdoba Province indicates an extension of the distribution of this genotype to the central region of Argentina (*4*). The year of introduction of genotype III remains unknown; previously, genotype VII strains CorAn9124 and CorAn9275 circulated in Córdoba Province (*4*).

The reasons for the reemergence of SLEV genotype III in Argentina 26 years after it was last detected are unknown. Possible associated factors are mosquito species communities' species composition and relative abundance, climate, and avian host abundance and immunity. No investigation was conducted until recently to elucidate the SLEV transmission cycles in Argentina. *Cx. quinquefasciatus* is probably an SLEV vector, according to studies of vector competence, population abundance of mosquitoes, and viral isolations in Argentina (*5*,*11*). Our isolation of SLEV from *Cx. quinquefasciatus* during the 2005 outbreak in Córdoba, and its higher abundance compared with other mosquito species, suggests its role as a vector in the urban transmission cycle of SLEV. The role of *Cx. interfor* as a SLEV vector is unknown. This report represents the first detection of SLEV-infected *Cx. interfor* mosquitoes. At this time, no epidemiologic data for arboviral diseases associated with this mosquito species have been reported.

High susceptibility of avian hosts in the city of Córdoba for SLEV infection was confirmed in 2004 when <1% of free-ranging wild birds circulated neutralizing antibodies (L. A. Diaz, unpub. data). The high neutralizing antibody titers we detected in chickens <3 months of age indicated recent infection and support the hypothesis that SLEV was responsible for the simultaneous outbreak.

While the specific avian amplifying hosts involved in the Córdoba outbreak remain unknown, important amplifiers would include competent reservoir hosts that are abundant and frequently exposed to infectious mosquito bites (*12*). Based on abundance alone, some possible candidates for avian reservoirs in Córdoba would include chickens, eared doves (*Zenaida auriculata*), Picui ground doves (*Columbina picui*), house sparrows (*Passer domesticus*), rufous horneros (*Furnarius ruffus*), great kiskadee (*Pitangus sulfuratus*), and others. Eared doves are competent amplifying hosts (*13*). Our study indicated high exposure rates in chickens. While adult chickens are generally incompetent for SLEV strains, higher viremia levels develop in baby chicks, which would probably be competent hosts (*14*).

Finally, the reemergence of SLEV in Córdoba represents an opportunity to study the ecology of this virus.

Table. Saint Louis encephalitis virus neutralizing antibody titers detected in domestic birds*

Host	Samples P/T	NtAb prevalence (%)	Age (mo)	Range in NtAb titer
Anser anser (goose)	4/5	80	>12	1,280†
Gallus gallus (chicken)	3/3	100	<3	1,280†
	8/13	62	>12	20–1,280

*At residence of a patient with Saint Louis encephalitis, during outbreak in Córdoba, Argentina, February 2005. P/T, number of positive samples/total samples analyzed; NtAb, neutralizing antibodies.
†There was no NtAb range in these 2 cases. All positive sera had the same titer.

Further studies are needed on vector competence for local strains of *Cx. quinquefasciatus* and *Cx. interfor* and on the reservoir competence of the bird species mentioned above.

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Human Parainfluenza Type 4 Infections, Canada

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During the fall/winter season of 2004–05, we found 9 respiratory specimens positive for human parainfluenza virus type 4 (HPIV-4) in our laboratory (43% of all HPIVs) from patients with mild to moderate respiratory illnesses. Sequencing studies identified 8 different HPIV-4A strains and 1 HPIV-4B strain.

Human parainfluenza viruses (HPIVs) have been recognized as a cause of respiratory tract infections for many decades. They belong to the *Paramyxoviridae* family, subfamily Paramyxovirinae, and are classified into 4 serotypes. Serotype 4 can be further subdivided into 2 antigenic subtypes, HPIV-4A and HPIV-4B (*1*). Although the epidemiology and clinical manifestations of serotypes HPIV-1 to HPIV-3 are well described, much less is known about HPIV-4, including its seasonality (*2*).

HPIV-4 has been mostly associated with mild illnesses (*3*,*4*). However, some evidence has indicated that it can cause more severe infections in some settings (*5*–*10*). We sought to describe the virologic and molecular characteristics as well as the clinical manifestations associated with HPIV-4 infections at our hospital during 2004–05.

The Study

From October 20, 2004, to March 8, 2005, we found 9 respiratory specimens positive for HPIV-4 in our virology laboratory. Specimens were collected from patients who were either admitted to a tertiary care hospital, seeking treatment at its emergency room, or attending an outpatient clinic connected to that hospital.

Specimens were placed into 96-well plates seeded with 8 cell lines and onto 2 shell vials (Table 1). Viral cultures were incubated for 21 to 24 days. For LLC-MK2 and MDCK cells, a hemadsoption test was performed at the end of the incubation period. Cytopathic effects (CPEs) or positive hemadsorption tests were confirmed by using immunofluorescence assays performed with monoclonal antibodies against HPIV-4 (Chemicon International, Temecula, CA, USA).

Viral RNA was extracted from culture supernatants by using the MagaZorb RNA Mini-prep kit (Cortex Biochem, San Leandro, CA, USA) and then tested with RT-PCR by using the Qiagen one-step RT-PCR kit. Amplicons of the fusion (F; 1631 nt) and hemagglutinin-neuraminidase (HN; 1721 nt) genes were generated by using primers listed in Table 1. Nucleotide sequences of all HPIV-4 strains were determined and entered into a multiple alignment generated by the Clustal W software (version 1.83)(*11*). Phylogenetic analyses were performed using distance methods with the PAUP software (version 4.0b10; Sinauer Associates, Sunderland, MA, USA).

Positive HPIV-4 samples consisted of 7 nasopharyngeal aspirates and 2 nasopharyngeal or throat swab specimens. The 9 viruses grew only in LLC-MK2 cells with all but one demonstrating CPEs from 12 to 21 days post-inoculation (mean: 19 days). The CPEs consisted of large and round swollen cells that progressed to destruction of the monolayer, without syncytium formation. The hemadsorption test result was positive for all isolates with subsequent confirmation by immunofluorescence staining. No other virus grew on the other cell lines. Bacterial cultures were done for 4 patients, and 1 culture was positive for *Streptococcus pneumoniae*.

Between October 20, 2004, and March 8, 2005, 1,424 respiratory specimens were submitted to our virology laboratory for viral culture. Of these, 371 (26%) were positive for a virus. HPIV-4 was the most frequent HPIV with 9 isolates, representing 43% of all HPIVs and 2.4% of all positive cultures. During the same period of 2003–04, we isolated only 1 HPIV-4 (2% of all HPIVs and 0.3% overall). In 2002–03, eleven HPIV-4 were recovered (23% of all HPIVs and 7.0% overall). Finally during the 2001-02 season, we found 3 HPIV-4 (15% of all HPIVs and 0.8% overall) (Figure 1).

The 9 HPIV-4 positive patients consisted of 6 children (5 <6 months of age) and 3 adults (Table 2). Among the pediatric patients, 3 had bronchiolitis, and the youngest (1.5 months) required a stay in the intensive care unit because of apnea. Two of the 3 patients with bronchiolitis had paroxysmal cough, and pertussis was suspected. The other 3 patients had upper respiratory tract illnesses. Among those 6 patients, 2 received antimicrobial drugs, and all recovered.

Among the 3 adults, 1 was 25 years of age, and 2 were >80 years of age. The former had pharyngitis and buccal ulcerative lesions. No other viral or bacterial pathogen was found. An 84-year-old woman was admitted to the hospital for severe bronchospasm and suspected pulmonary

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Table 1. Cell lines used for viral culture and primers used for HPIV-4 PCR testing

Cell lines	Oligonucleotide sequences (5′–3′)	Target genes
Mink lung Human foreskin fibroblast Human lung carcinoma (A-549) Vero Hep-2 Human rhabdomyosarcoma (RD) Transformed human kidney 293 Human colon adenocarcinoma (HT-29) Madin-Darby canine kidney (MDCK) Tertiary monkey kidney (LLC-MK2)	ATGGGTGTCAAAGGTTTATC (forward) AATTATGCAGATTGTAACTGTC (reverse) ATGGTGAAAAGAACATGGAG (forward) TGGAGTATCCAGCAGTAAGA (reverse)	Fusion Hemagglutinin-neuraminidase

edema. In addition, a 90-year-old woman had a flulike illness but antigenic test results were negative for influenza. The 3 patients had no complications and survived. Overall, 4 of the 9 patients were hospitalized with a mean length of stay of 8 days (range 2–14 days). Six (66%) of the patients had an underlying disease.

Phylogenetic trees of the F and HN genes were similar. Eight different HPIV-4 isolates clustered with the HPIV-4A reference strain, whereas the remaining one clustered with the HPIV-4B reference strain (Figure 2). The percentages of nucleotide (nt)/amino acid (aa) identity for the 8 HPIV-4A strains were 97%/100% (F gene) and 97%/99% (HN gene). In contrast, the percentages of nt/aa identity between the HPIV-4A strains and the HPIV-4B isolate were 89%/92% (F gene) and 86%/87% (HN gene).

Conclusions

HPIV-4 is considered a rare pathogen because it has been isolated only occasionally from respiratory tract specimens, although seroprevalence studies have shown that 70%–90% of young adults have specific antibodies against it (*12,13*). Lately, a few reports have shown that HPIV-4 might be more frequent than previously thought when sensitive RT-PCR methods are used (*5,8,10,14*). Using viral cultures, we found that HPIV-4 accounted for 43% of all HPIVs isolated in our laboratory during the 2004-05 fall and winter seasons. Direct testing of clinical specimens with RT-PCR would have likely resulted in higher detection rates.

Possible explanations for the rarity of HPIV-4 are in part related to its slow growth in LLC-MK2 cells, a cell line not used in most virology laboratories. Also, CPEs are not always present (may take 2–3 weeks to appear), and the hemadsorption reaction is occasionally weak (*1*). Finally, symptoms associated with HPIV-4 are generally mild and do not elicit requests for a cell culture.

To our knowledge, no seasonality has been described for HPIV-4. In our area, HPIV-4 was isolated every year during the last 4 years with peaks of activity occurring every other year, similar to HPIV-1 and 2 (*15*). The biennial pattern of HPIV-4 would require confirmation in larger studies from other countries. In temperate countries, the virus is usually recovered during the late fall and winter seasons (*2,13,14*). In fact, over the last 4 years, only 4 HPIV-4 isolates were recovered outside our study period, 3 in April and 1 in May.

The retrospective aspect of our study and the small number of viral isolates limit definitive conclusions on the clinical manifestations of HPIV-4 infections. We note that young children were preferentially affected as previously reported (*2,5–7,14*) although they also constitute the most likely population for whom viral cultures would be obtained. In children, clinical conditions included upper respiratory tract infections, bronchiolitis, and pertussislike clinical syndromes (*7*). Infected adults had various clinical presentations, i.e., pharyngitis, bronchospasm, and flulike illnesses, although further work is required to describe the full clinical spectrum of HPIV-4 infections.

Our 9 HPIV-4 isolates could be further subdivided into 8 different HPIV-4A and 1 HPIV-4B strains, according to sequences obtained from the 2 glycoproteins F and HN. Reports of HPIV-4B infection have been infrequent in the last 2 decades, similar to our findings (*9*).

In summary, HPIV-4 infections can be relatively common during the fall and winter seasons of some years and

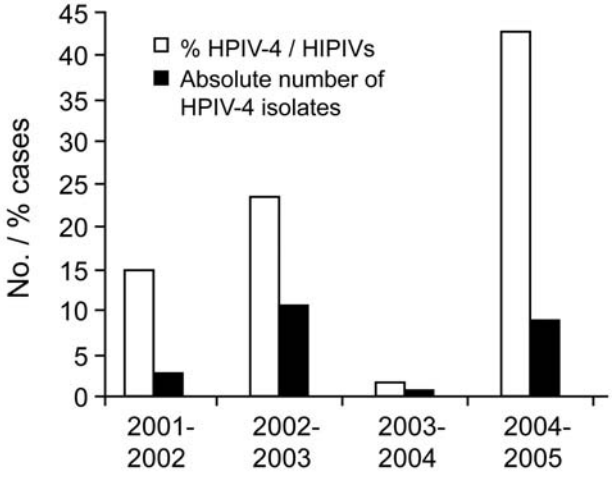

Figure 1. Seasonality of human parainfluenza virus type 4 (HPIV-4) infections during fall and winter of 4 consecutive years.

Table 2. Clinical data of 9 patients with HPIV-4 infections in Quebec City, Canada*

Patient	Sample type (date of collection)	Age/sex	Underlying Illness	Symptoms and signs	Hospitalization (d)	Final diagnosis
1	NPA (2004 Dec 5)	1.5 mo/M	–	Cough, apnea, fever, low O_2 sat.	Yes, ICU (3)	Bronchiolitis
2	NPA (2004 Oct 20)	2.5 mo/M	Premature (32 wk), POF, PPS	Cough, apnea, low O_2 sat.	Yes (14)	Bronchiolitis
3	NPA (2004 Nov 2)	3 mo/M	–	Cough, apnea, BOM	Yes (2)	URTI and BOM
4	NPA (2005 Jan 4)	5 mo/M	Premature (33 wk)	Cough, fever	No	URTI
5	NPA (2005 Jan 19)	6 mo/F	Premature (26 wk), PD	Rhinorrhea, wheezing	No	Bronchiolitis
6	NPA (2004 Nov 18)	2.7 y/F	Asthma	Rhinorrhea, cough, wheezing	No	Sinusitis and bronchospasm
7	TS (2005 Mar 8)	25 y/M	-	Fever, right tonsil ulcerative lesions	No	Viral pharyngitis
8	NPA (2005 Jan 25)	84 y/F	CHD, COPD	Dyspnea, low O_2 sat., cyanosis	Yes (14)	PE, MI, and persisting bronchospasm
9	NPS (2005 Jan 21)	90 y/F	AF, dementia	Fever, muscle aches	No	Flulike syndrome

*HPIV-4, human parainfluenza virus type 4; NPA, nasopharyngeal aspirate; O_2 sat., oxygen saturation; ICU, intensive care unit; POF, permeable oval foramen; PPS, peripheral pulmonary stenosis; BOM, bilateral otitis media; URTI, upper respiratory tract infection; PD, pulmonary dysplasia; TS, throat swab; CHD, coronary heart disease; COPD, chronic obstructive pulmonary disease; PE, pulmonary edema; MI, myocardial infarction; NPS, nasopharyngeal swab; AF, atrial fibrillation.

are probably underdiagnosed due to their fastidious growth. Detection of this respiratory pathogen needs to be improved through rapid molecular assays.

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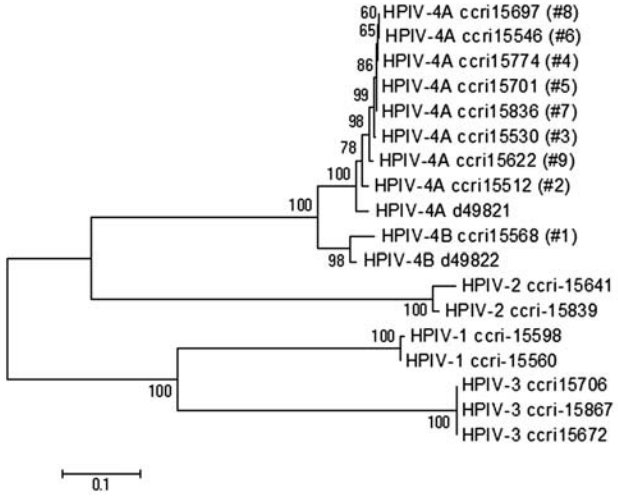

Figure 2. Phylogenetic analysis based on fusion (F) gene nucleotide sequences from clinical and reference human parainfluenza virus (HPIV) strains. The tree was built by using distance method and the neighbor-joining algorithm with Kimura 2 parameters. The topologic accuracy of the tree was evaluated by using 500 bootstrap replicates. Strains isolated at the Research Center in Infectious Diseases (Quebec, Canada) are indicated by a specific identification number (ccri) followed by the patient number in parentheses. Public sequences for HPIV-4A (GenBank accession no. d49821) and HPIV-4B (GenBank accession no. d49822) strains are also indicated in the tree.

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Methicillin-Resistant *Staphylococcus aureus* at Canoe Camp

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We investigated a cluster of community-associated methicillin-resistant *Staphylococcus aureus* infections among persons at a wilderness canoe camp. Isolates from the investigation had identical profiles for susceptibility, pulsed-field gel electrophoresis, and toxins. Participants in activities that involve skin injury, person-to-person contact, and inadequate hygiene are at increased risk for methicillin-resistant *S. aureus* infections.

In 2000, the Minnesota Department of Health (MDH) initiated prospective active surveillance at 12 sentinel hospitals for community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections. Cases are classified as CA-MRSA if the patient lacks traditional healthcare-associated risk factors (hospitalization, surgery, dialysis, residence in a long-term care facility during the year before culture, or having an indwelling device at the time of culture). Although >80% of CA-MRSA infections involve skin or soft tissue, invasive disease that involves bones, joints, and sepsis can occur (*1*,*2*). During 2000–2004, 2% of CA-MRSA cases reported to MDH were joint infections.

Outbreaks of CA-MRSA have been associated with sports that require physical contact and result in frequent damage to skin (*3*,*4*) and with crowded settings (e.g., correctional facilities, military settings), where access to hygiene measures is limited (*5*,*6*). In response to a case cluster of CA-MRSA infections at a wilderness canoe camp, MDH performed an investigation to identify additional cases; MRSA colonization among staff, campers, and household members; and risk factors for infection.

The Study

In August 2004, MDH was notified of 2 previously healthy 15-year-old male patients who were hospitalized for septic arthritis caused by MRSA; 1 had required treatment in an intensive care unit. The patients had been in the same group that participated in a 21-day wilderness canoe trip in northern Minnesota, USA. A third case-patient, a previously healthy 17-year-old female camper who had participated in an earlier 21-day trip and had had a skin infection without complications, was identified during the investigation (Table).

A case was defined as a clinically relevant, culture-confirmed, MRSA infection that occurred between June 1 and September 30, 2004, in a staff member, camper, or member of a camper's household. Colonization was defined as MRSA identified from the nares of these persons in the absence of MRSA infection.

MDH and local public health staff investigated the base camp. During the 21-day canoeing trip, participants spent 1 day at base camp at the beginning and end of the trip; the remaining time was spent canoeing, portaging, and camping on the trail. Camping groups were composed of 5–6 campers and a guide. Campers were of the same sex and were 15–17 years old. Campers and guides shared a common tent and did all activities together, but each had his or her own sleeping bag and towels. Campers and guides did not have access to running water or soap outside of base camp; the information on the wilderness area permit discourages use of soap, including biodegradable soap, within 150 feet of water.

Campers who had participated in the same, concurrent, or preceding canoe trips with the same itinerary and their household members were interviewed regarding health history, skin infections and injury, hygiene, clothing, camping behavior, and contact with other campers. Swabs of anterior nares were obtained from consenting staff, campers, and members of campers' households and were submitted to the MDH Public Health Laboratory. Isolates were identified by standard methods. Susceptibility testing was conducted by broth microdilution (PML Microbiology, Wilsonville, OR, USA) and interpreted according to Clinical and Laboratory Standards Institute (formerly NCCLS) criteria. Pulsed-field gel electrophoresis (PFGE) was performed by digestion with the restriction enzyme *Sma*I (*7*). Patterns were compared visually and with Bionumeric software (Applied Maths, Kortrijk, Belgium) to reference strains of *S. aureus* (*8*). Sequences encoding toxin genes (TSST-1, Panton-Valentine leukocidin, *sea*, *seb*, *sec*, and *sed*) were detected by PCR (*9*,*10*). Univariate analysis was conducted with EpiInfo version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA, USA) using a significance level of $p<0.05$.

Of 21 campers, 19 (90%) were interviewed. The 2 case-patients had occasionally shared the same canoe. All campers reported having had frequent skin injuries, including insect bites, cuts, burns, blisters, and abrasions, during their trips. Campers reported having worn shorts most of

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Table. Methicillin-resistant *Staphylococcus aureus* infections, canoe camp, Minnesota, USA, 2004*

Case-patient	Clinical progression
1	Reported mosquito bite on knee; evacuated from camp because of knee injury. Airlifted from clinic to hospital because of septic shock; required supportive care and knee debridement. Empirically treated with intravenous clindamycin. Had MRSA-positive joint culture and surgical specimens from knee, negative blood cultures. Treated with clindamycin, nafcillin, vancomycin, and linezolid. Hospitalized 11 d; had no long-term sequelae.
2	Knee stiffness and fever developed on the way home from camp. Did not report specific knee abrasion or injury. Admitted to hospital and underwent knee debridement. Empirically treated with intravenous cefazolin. Had MRSA-positive joint cultures and surgical specimens from knee. Treated with clindamycin. Hospitalized for 4 d; had no long-term sequelae.
3	Developed a forearm abscess 1 mo after trip. Did not report specific skin abrasion or injury. Skin culture was positive for MRSA. Treated with oral clindamycin. Was not hospitalized; had no long-term sequelae.

*MRSA, methicillin-resistant *Staphylococcus aureus.*

the time and frequently having had wet skin, clothing, and shoes. No differences were identified between campers with and without CA-MRSA or between camping groups with and without CA-MRSA in terms of health history, use of antimicrobial drugs during the past year, skin infections and injury, hygiene, clothing, or camping behavior. Interviews were also conducted with 55 camper household members, representing 19 households. Members of 2 households could not be reached, and no information was obtained on the number of persons in these households. Interview responses of household members of case-patients did not differ from those of household members of the other campers and staff. No case-patient or colonized person reported a history of MRSA infection or colonization. One camper who was not a case-patient reported having had a positive blood culture for CA-MRSA in the prior year; this camper had been hospitalized with an infection that began as cellulitis and resulted in septic shock and was found to be not colonized at the time of the investigation. Also, the camp director informed MDH that in the prior year, 2 counselors had been treated for apparent spider bites at a local clinic; however, cultures had not been obtained.

Nares swabs were obtained for case-patients 2 and 3 but not case-patient 1 because he was receiving nasal mupirocin treatment. None of the case-patients was colonized with MRSA. Nares swabs were also obtained for 62% of campers without MRSA infection; of these, 1 female camper who had participated in a preceding trip was found to be colonized with MRSA. Swabs obtained from the anterior nares of 16 camp guides and base camp staff members showed MRSA colonization in 1. This staff member had not gone on any camping trips with the case-patients. Of nares swabs from 40 household members (including all 8 household members of case-patients), none was positive for MRSA.

All 5 MRSA isolates (from the 3 case-patients and the 2 colonized persons) were susceptible to clindamycin, ciprofloxacin, erythromycin, gentamicin, linezolid, quinopristin/dalfopristin, rifampin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin and had an MIC for mupirocin of <4 μg/mL. The isolates were USA400 pulsed-field type and were indistinguishable by PFGE (*8*). The isolate from the camper who had had an MRSA infection in 2003 was also indistinguishable by PFGE. All 2004 isolates contained genes encoding Panton-Valentine leukocidin, *sea*, and *sec* (*10*).

To prevent further infections, camp staff were given information about *S. aureus*, MRSA, and infection-prevention measures. Campers were instructed to wear long pants when possible, protect skin from injury (e.g., use insect repellent), report all suspected skin infections promptly, keep skin as clean as possible, use alcohol-based hand sanitizers, and not share personal items like towels and clothing. Frequently touched surfaces were cleaned with a bleach solution, and life vests were assigned to individual campers and disinfected between campers.

Conclusions

CA-MRSA infections are increasingly reported and can be severe. Despite multiple outbreaks attributed to USA300, only a few reported case clusters have been caused by USA400 (*11*,*12*). USA400 does contain virulence factors, and although most cases have been mild skin and soft tissue infections, some have been severe and fatal (*13*). Furthermore, although the colonization rate was low

for staff, campers, and household members (2.7%), it was higher than CA-MRSA colonization rates for the general population (*14*), as would be expected for case-patient contacts. *S. aureus* (including CA-MRSA) spreads easily among people in crowded settings, particularly when adequate hygiene cannot be maintained, both of which occurred in this camping setting.

Although anecdotal, suspected, and confirmed cases during the prior year are consistent with ongoing transmission associated with the camp, that spider bites were reported is noteworthy because CA-MRSA infections are often misdiagnosed as spider bites (*15*). Injuries and breaks in skin should be closely monitored in settings where medical care is not readily available. Methods for skin protection and hygiene should be developed and implemented for populations in settings where CA-MRSA transmission and infection can be anticipated.

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Knowledge, Attitudes, and Practices of Avian Influenza, Poultry Workers, Italy

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We asked Italian poultry workers about knowledge, attitudes, and practices regarding avian influenza. It was perceived to be a low occupational hazard, and wearing protective equipment and handwashing were not routine practices. Knowledge of transmission and preventive measures should be improved. Employers and health professionals should provide more effective information.

Infection of poultry with influenza A (subtype H5N1) virus is responsible for outbreaks in birds and a human case-fatality rate of 58% (*1*). The most likely means of transmission is from infected birds to humans and from the environment to humans, but evidence for human-to-human transmission is limited (*2*). This virus can be transmitted if a person has direct contact with infected poultry or surfaces and objects contaminated by poultry droppings. Two epidemics caused by avian influenza virus H5 and H7 subtypes occurred in poultry in Italy from 1997 through 2001. A plan was recently developed for adequate response to influenza pandemics, and farmers, veterinarians, and healthcare workers have been educated about diagnosing, detecting, and preventing the spread of avian influenza (*3*).

Workers in the poultry industry, who commonly have contact with live, sick, or dying poultry, are at high risk for avian influenza. These workers are at increased risk because of food handling and preparation of raw poultry meat and products. Concern exists that avian influenza could be transmitted from uncooked birds or bird products to humans (*4*,*5*). This study evaluated knowledge, attitudes, and infection control practices of poultry workers in Italy regarding avian influenza.

The Study

A total of 284 poultry workers at 110 poultry farms throughout the Campania region of Italy were recruited into the study from December 2005 through March 2006. The workers were interviewed confidentially in their workplace regarding demographics, work activity, knowledge of transmission and prevention of avian influenza, attitudes toward this disease, compliance with precautions at work, and sources of information (online Appendix Figure, available in English and Italian from http://www.cdc.gov/ncidod/EID/vol12no11/06-0671_appG.htm). Multiple logistic regression analysis with adjusted odds ratios and 95% confidence intervals and multiple linear regression analysis with adjusted β coefficients were performed with Stata software (*6*).

A total of 257 poultry workers were interviewed (response rate 90.5%.). Average age was 43 years (range 19–75 years), average duration of work activity was 18 years, and median number of daily exposures to breeder animals was 18,500. One third of the workers had a high school or college education.

Of the 257 workers, 63.8% correctly defined avian influenza as a contagious infection caused by a virus that can affect all species of birds (Table 1), and 21.8%–81.7% knew that avian influenza can be transmitted by touching uncooked eggs or infected animals. Nearly all workers identified poultry and wild birds as common vectors. Most knew that poultry workers had a high risk of being infected and that butchers and veterinarians had a lower risk. Only 22.6% provided a correct definition of this disease and knew routes and vectors of transmission.

Table 1. Knowledge of avian influenza among 257 poultry workers, Italy

Variable	Correctly answered, no. (%)
Definition (contagious infection caused by virus that can affect all species of birds)	164 (63.8)
Modes of transmission	
Animal to human	210 (81.7)
Animal to animal	206 (80.2)
Environment to human	153 (59.5)
Eating uncooked poultry	149 (58)
Eating uncooked eggs	102 (39.7)
Touching uncooked poultry	88 (34.2)
Touching uncooked eggs	56 (21.8)
Touching wild birds	246 (95.7)
Touching poultry	232 (90.3)
Touching saliva, nasal secretions, feces, and fomites of infected birds	167 (65)
Risk groups	
Poultry workers	194 (75.5)
Butchers	134 (52.1)
Veterinarians	108 (42)
Use of preventive measures	
Face mask	176 (68.5)
Gloves	158 (61.5)
Outer garments	157 (61.1)
Boots or boot covers	144 (56)
Eye protection	111 (43.2)
Handwashing with soap and water	161 (62.7)

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Knowledge was greater in persons with more education, those who worked a longer time, those who believed they were at high risk of contracting avian influenza, and those who needed information (Table 2). With respect to identifying measures that protect poultry workers from exposure to avian influenza, correct responses ranged from 34.2% for all protective measures to 43.2% for eye protection and 68.5% for face masks. Greater knowledge was observed in those who received information from health professionals and employers, those who believed they were at high risk, and those who worked only with poultry (Table 2).

Most poultry workers believed that avian influenza was a serious (69.7%) but preventable (70.8%) disease. Mean

Table 2. Logistic and linear regression models results of knowledge, attitudes, and practices of avian influenza among 257 poultry workers, Italy*

Variable	OR	95% CI	p value
Model 1: General knowledge about avian influenza; log likelihood –124.45, χ^2 25.58, df 6, p = 0.0003			
Years of working activity	1.03	1.01–1.06	0.015
Need of additional information	2.59	1.17–5.72	0.019
Perception of risk for avian influenza	1.13	1.01–1.26	0.037
Education level	1.63	1.02–2.61	0.042
Avian influenza is a serious and preventable disease	1.44	0.72–2.88	0.31
No. breeder animals exposed to per day	0.87	0.65–1.16	0.33
Model 2: Knowledge of all measures that protect poultry workers from exposure to avian influenza; log likelihood –142.95, χ^2 44.40, df 7, p < 0.0001			
Health professionals and employers as sources of information	3.62	1.99–6.59	<0.001
Perception of risk for avian influenza	1.19	1.07–1.34	0.002
Working with poultry and eggs	1.0†	–	–
Working with only poultry	2.11	1.17–3.81	0.013
Education level	1.37	0.90–2.08	0.14
General knowledge of avian influenza	1.44	0.74–2.8	0.28
No. breeder animals exposed to per day	0.88	0.68–1.14	0.33
Hours worked per day	1.05	0.95–1.14	0.35
Model 3: Modification of working habits in the past 3 mo; log likelihood –119.48, χ^2 42.72, df 10, p < 0.0001			
Health professionals and employers as sources of information	0.34	0.17–0.69	0.003
Marital status	0.31	0.13–0.76	0.01
Age	0.96	0.92–0.99	0.018
Avian influenza is a serious and preventable disease	2.29	1.09–4.76	0.028
General knowledge of avian influenza	2.11	1.03–4.31	0.041
Poultry workers are a risk group	2.30	0.94–5.65	0.07
Perception of risk for avian influenza	1.09	0.97–1.22	0.15
Working with poultry and eggs	1.0†	–	–
Working with only poultry	0.64	0.03–1.34	0.24
Sex	1.45	0.73–2.86	0.29
Education level	1.32	0.78–2.22	0.3
Model 4: Preventive measures behavior; log likelihood –119.36, χ^2 45.26, df 4, p < 0.0001			
Knowledge of preventive measures	5.95	3.06–11.56	<0.001
Working with poultry and eggs	1.0†	–	–
Working with only eggs	0.28	0.11–0.73	0.009
General knowledge of avian influenza	1.48	0.72–3.02	0.28
Health professionals and employers as sources of information	1.37	0.70–2.69	0.37
Variable	β coefficient	t	p value
Model 5: Perception of risk for avian influenza; F(7,249) = 8.25, p < 0.0001, R^2 18.8%, adjusted R^2 16.6%			
Working with poultry and eggs	–†	–	–
Working with only poultry	–1.72	–5.0	<0.001
Hours worked per day	–0.19	–3.74	<0.001
Need additional information	1.16	3.56	<0.001
Media and television as sources of information	2.02	3.36	0.001
Knowledge of preventive measures	0.77	2.36	0.019
No. children	–0.21	–1.56	0.12
Sex	–0.32	–0.9	0.37
Constant	3.42		

*OR, odds ratio; CI, confidence interval; df, degrees of freedom.
†Reference category.

total scores (scale of 1 to 10) for perceived risk of contracting avian influenza during work activity and for co-workers and family members were 3.2 and 3.1, respectively, which indicated low-risk perception. Only 4.3% showed great concern about risk. Respondents who were more likely to believe that they were at high risk worked fewer hours, knew protective measures for exposure to avian influenza, had received information from the mass media, and needed information. Workers who were exposed only to poultry were less likely to perceive risk (Table 2).

A total of 23.7% reported that in the past 3 months they had modified their work habits because of fear of contracting avian influenza. Those more likely to modify their behavior were younger, married, had more knowledge of avian influenza, believed that it was a serious but preventable disease, and received information from sources other than health professionals and employers (Table 2).

Regarding compliance with precautions to avoid spreading virus through food while working, 59.9% routinely washed their hands and disinfected surfaces and utensils that had been in contact with raw meat. Wearing personal protective equipment was not a routine practice because 82.9% always wore outer garments, 82.9% wore boots or protective boot covers, 59.9% wore gloves, 59.9% wore face masks, 24.5% wore eye protection, and 87.9% washed their hands. A total of 24.1% always wore protective clothing and washed their hands; these practices were more common in poultry workers who knew that these measures were protective and less common by workers who handled only eggs (Table 2).

All poultry workers had received information regarding avian influenza. The most common sources were mass media (91.8%), health professionals (47.5%), and employers (6.2%); 62.3% wanted more information.

Conclusions

Knowledge of avian influenza can be improved, as shown in a study of consumers (G. Di Giuseppe et al., unpub. data). We expected to find more knowledge in educated poultry workers, especially in how to identify potential animal cases and minimize risk for transmission. However, our observations indicate that information is not correctly disseminated because those who receive information from the mass media and who need information were more likely to have a perception of high risk. Therefore, tailored educational programs, including booklets and seminars, could be beneficial in improving self-risk assessment of poultry workers.

Two thirds of poultry workers believed that avian influenza is a serious and preventable disease, but the study showed a perception of low risk of contracting this disease at work because only 4.3% indicated strong concern, although the largest percentage correctly recognized that they are a risk group. Workers who handled only poultry were less likely to perceive a risk than those who handled other products.

Studies have identified direct exposure to infected poultry as the primary risk factor in transmission of avian influenza virus to humans. A cohort study of poultry workers in Hong Kong showed that greater exposure to poultry was associated with antibody to H5 hemagglutinin (*4*). In Thailand, a case-control study showed that activities involving exposure to poultry were associated with influenza caused by H5N1 virus (*7*). In Vietnam, a population study in a rural area with outbreaks of highly pathogenic avian influenza showed a dose-response relationship between poultry exposure and illness (*8*).

Low adherence to the recommendations of the World Health Organization to avoid spread of avian influenza through food while working has been reported; use of protective clothing and handwashing is inadequate (*9*). In our study, there was a subset of workers who routinely followed guidelines because lower compliance was observed in those working only with eggs. Moreover, those who did not know precautions had a 6-fold greater risk for inconsistent adherence to preventive guidelines compared with those who identify them.

Improving knowledge of transmission and application of preventive measures is a useful public health strategy for reducing the effects of avian influenza in poultry workers. Employers and health professionals should work together to provide effective and coordinated information to these workers.

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Avian Influenza H5N1 Screening of Intensive Care Unit Patients with Community-acquired Pneumonia

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From February 1, 2005, to January 31, 2006, we screened 115 adults for avian influenza (H5N1) and influenza A if admitted to an intensive care unit with pneumonia. Using reverse transcription-PCR, viral culture, and serologic testing for anti-H5 antibody, we identified 8 (7%) patients with influenza A (H3N2); none had H5N1. Estimated costs for H5N1 screening were $7,375.

The ongoing avian influenza (H5N1) pandemic poses risks to both human and animal health (*1–5*). The potential exists for cross-species transmission of avian influenza to humans and subsequent reassortment of avian and human influenza viruses in coinfected persons (*6*). Although atypical presentations of avian influenza (H5N1) have been reported (*7*,*8*), in most H5N1 case-patients pneumonia was the primary condition (*3*,*4*). To assess the prevalence of avian influenza (H5N1) and influenza A pneumonia, we screened adults admitted to a medical intensive care unit (ICU) with community-acquired pneumonia (CAP) for H5N1 and calculated the cost estimates for H5N1 screening in a tertiary care center of an H5N1-endemic area in Thailand.

The Study

Thammasat University Hospital is a 450-bed tertiary care center with an 8-bed intensive care unit (ICU) equipped with central air-conditioning and 2 isolation rooms. The hospital serves a 150-km radius referral base in central Thailand and has 980 healthcare workers (HCWs). Annual influenza vaccination was not routinely offered to HCWs. During the study period, 2 confirmed cases of H5N1 occurred within 150 km of our hospital.

All adults admitted to the ICU with CAP between February 1, 2005, and January 31, 2006, were eligible for enrollment. Tracheal aspirates were collected for H5N1 testing, with reverse transcription (RT)-PCR, and viral culture. In patients <60 years with >14 days survival posthospitalization, paired acute-phase and convalescent-phase serum specimens were collected for identifying anti-H5 antibody. Acute-phase serum specimens for determining anti-H5 antibody were obtained within 1 week of symptoms, while convalescent-phase serum specimens were obtained >14 days after the acute-phase specimens were collected. Data collection included demographic characteristics, clinical data, and the costs associated with H5N1 screening. The diagnosis of CAP was defined according to the criteria recommended by the American Thoracic Society (*9*). Patients who were hospitalized for >2 days and in whom pneumonia developed were excluded from this study. The current Thai national surveillance definition for probable avian influenza (H5N1) included the following: 1) presence of fever (>38°C), and 2) influenza-like illness, and 3) exposure to sick poultry or residence in the disease-endemic areas with excess poultry death rates, and 4) radiographic evidence of severe CAP without an identified etiologic agent (*10*).

Viral cultures for H5N1 and influenza A, as part of screening, were incubated in Madin-Darby canine kidney (MDCK) cell monolayers at the Thai National Institute of Health. Tracheal aspirate specimens were tested by an RT-PCR assay specific for the hemagglutinin gene of H5 (*11*). If a specimen yielded a positive H5 band, the specimens were confirmed by different RT-PCR primers and by real-time RT-PCR (*12*). All serum samples were tested for H5-specific antibody by a microneutralization (micro-NT) test. The reactive samples underwent confirmatory immunofluorescence testing by using H5-transfected 293 T cells as the test antigen (*13*). Influenza A/Thailand/1(KAN-1)/2004 (H5N1) was used as the test virus. Acute-phase and convalescent-phase serum samples were serially diluted from 1:20 to 1:80. On the basis of previously established criteria, a positive test was defined as a neutralizing antibody titer >80 with a confirmatory immunofluorescence assay (*14*). Adults ≥60 years of age were excluded from the serologic tests because the H5N1 micro-NT was previously reported to be less specific in this population (*14*).

Laboratory diagnostic costs (RT-PCR for H5N1, viral culture, and paired acute- and convalescent-phase serum samples for anti-H5 antibody) for each patient were obtained from line-item reports of the hospital's fiscal system. All costs in Thai baht currency were converted to US dollars at an exchange rate of 40 bahts per 1 US dollar. The

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cost for isolation of the index case, if influenza A or avian influenza (H5N1) was detected, were calculated from prior cost estimates (*15*).

One hundred fifteen of 450 patients (25%) met the definition of CAP and consented to study participation. The patient characteristics are summarized in Table 1. None of the 115 patients had tracheal aspirates positive for H5N1; also not positive were any serologic test results from the 42 patients (37%) who were <60 years old and survived >14 days after hospitalization. We were unable to calculate the prevalence of anti-H5 antibody in this sample, given that only 37% of participants underwent complete diagnostic antibody testing.

Eighteen patients (16%) met the Thai national surveillance definition of probable H5N1, yet tracheal aspirates and serologic test results were negative for H5N1. The median time from initial symptoms to hospitalization was 4 days (range 2–8 days), and all 18 were appropriately placed on contact and droplet isolation; the mean duration of isolation was 9 days (range 4–13 days).

Although 48 (42%) of the 115 participants had no identified etiologic agent associated with CAP, *Streptococcus pneumoniae* (n = 39; 34%), influenza A (H3N2) (n = 8; 7%), *Staphylococcus aureus* (n = 7; 6%), and *Haemophilus influenzae* (n = 6; 5%) were the most common microorganisms detected. In addition, 19 patients (n = 19; 16%) had gram-negative microorganisms detected. All patients with H3N2 pneumonia were promptly transferred to an isolation room; 5 (62.5%) had dual infections of H3N2 and *S. aureus* (n = 3), *Klebsiella pneumoniae* (n = 1) and *Pseudomonas* species (n = 1), while CAP due to H3N2 developed in 3 (37.5%). Of 18 patients who met the definition of probable H5N1, 8 (44.5%) had *S. pneumoniae* infection, 4 (22.5%) had *S. aureus* infection, 2 (11%) had H3N2 infection, 2 (11%) had *Burkholderia pseudomallei* infection, and 2 (11%) had no other agent detected. No CAP patients had anti-H5 antibody seroconversion, although 1 participant had evidence of positive anti-H5 antibody with low titer (10) during the recovery phase. This patient lived in an avian influenza (H5N1)–endemic

Table 1. Demographic and clinical data for 115 hospitalized adults with severe community-acquired pneumonia at a tertiary care center in an H5N1-endemic region of Thailand*

Characteristics	Total (N = 115)	Influenza A H3N2 (n = 8)	Without concomitant influenza A H3N2 (n = 107)	p value†
Age, years (mean, range)	64 (17–82)	72 (55–82)	64 (17–74)	0.06
Sex, male	48 (42)	4 (50)	44 (41)	NS
Tobacco smoking	21 (18)	1 (12)	20 (19)	NS
No. of comorbid conditions (median, range)	1 (0–4)	3 (1–4)	1 (0–3)	<0.001
Underlying diseases‡§				
Lung disease	48 (42)	4 (50)	44 (41)	NS
Diabetes	25 (22)	2 (25)	23 (21)	NS
Cardiovascular	14 (12)	1 (12)	13 (12)	NS
Cerebrovascular or other neurologic disease	12 (10)	1 (12)	11 (10)	NS
Other	42 (37)	3 (38)	39 (36)	NS
Initial clinical symptoms§				
Pulmonary¶	108 (94)	7 (87)	101 (94)	NS
Gastrointestinal#	8 (7)	4 (50)	4 (4)	0.001
Neurologic**	9 (8)	1 (12)	8 (8)	NS
Other	2 (2)	0 (0)	2 (1)	NS
APACHE-II score, median (range)	16 (9–22)	17 (9–22)	15 (9–22)	NS
History of recent travel	0	0	0	NA
Met definition of probable H5N1	18 (16)	2 (25)	16 (15)	NS
History of exposure to index case	0	0	0	NA
Outcome				
Death††	12 (10)	7 (88)	5 (5)	<0.001
LOS in MICU	14 (1–46)	15 (1–46)	14 (2–42)	NS
H5N1 seroconversion	0	0	0	NA

*Data are no. (%) of patients, unless otherwise indicated; NS, nonsignificant; NA, nonapplicable; APACHE-II score, Acute Physiology and Chronic Health Evaluation Score II; LOS, length of stay; MICU, medical intensive care unit.
†Categorical variables were compared using χ^2 or Fisher exact test, as appropriate; Continuous variables were compared using the Wilcoxon rank sum test or *t* test, as appropriate. All p values were 2-tailed; p<0.05 was considered significant.
‡Included those considered by the Advisory Committee on Immunization Practices of the Centers for Disease Control and Prevention to be associated with an increased risk of complication from influenza infection.
§Most patients had multiple underlying diseases and initial clinical symptoms so the sums of all percentages are>100%.
¶Included cough, dyspnea or tachypnea, rigor and/or chills, pleuritic chest pain, purulent sputum, or changes in the characteristics of sputum, and auscultatory findings.
#Included diarrhea, and/or nausea or vomiting, abdominal tenderness.
**Included drowsiness, confusion, coma.
††All patients did not receive antiviral therapy.

area without a documented excess poultry death rate, and reported no exposure to sick poultry or persons with suspected avian influenza (H5N1) infection. His tracheal culture yielded *S. pneumoniae*. All patients with H3N2 pneumonia sought treatment between late March and November, the influenza A season in Thailand, and were in contact and droplet isolation for a mean of 7 days (range 1–12 days). The all-cause mortality rate was 10% (Table 1). The cost estimates were $7,375 for H5N1 screening, $23,328 for subsequent infection control measures, $300 for annual influenza vaccination of ICU HCWs, and $9,800 for annual influenza vaccination of the entire hospital staff (Table 2). The perceived benefits of vaccination of all ICU HCWs included reduced risk for influenza among vaccinated HCWs and reduced risk for influenza transmission to at-risk ICU patients.

Conclusions

Our study findings are relevant to the prevention and control of spread of both H5N1 and H3N2. The relatively high prevalence of H3N2 (7%) among our CAP patients suggests that HCWs in ICUs in disease-endemic regions are at high-risk of acquiring influenza A. An annual influenza vaccination occupational health program, similar to those in developed countries, along with targeted case identification of patients at high risk for influenza pneumonia, may help minimize the clinical and economic consequences of influenza A transmission. Although the importance of a single patient's positive low-titer anti-H5 antibody in this study was uncertain, this finding may represent a false-positive test, given that the patient had no notable exposure to sick poultry or to persons with suspected H5N1 infection. Additionally, the fact that all 18 probable case-patients had negative results for H5N1 suggests that the current Thai surveillance definition may need further refinement. Given the potential for reassortment of H5N1 and influenza A in a coinfected person residing in a disease-endemic setting, additional H5N1 screening, along with cost-effectiveness studies, are warranted before this screening strategy is adapted to H5N1-endemic areas.

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Table 2. Cost estimates for routine avian influenza (H5N1) surveillance, laboratory diagnostics, and infection control measures in the ICU, February 1, 2005 – January 31, 2006*

Category	No. measures	Estimated cost (US$)	Total (US$)
Cost associated with H5N1 routine screening			
Diagnostic testing			
RT-PCR	115	$25 × 115	2,875
Viral culture	115	$30 × 115	3,450
Paired acute- and convalescent-phase serology for anti-H5 antibody†	42	$25 × 42	1,050
Isolation for probable H5N1 (n = 18)‡			
Gowns/d	1,800	$1/gown × 1,800 × 9 d	16,200
Gloves/d	1,800	$0.05/pair × 1,800 × 9 d	810
Surgical masks/d	1,800	$0.25/mask × 1,800 × 9 d	4,050
Staff time (min/d) to put on/take off gloves, gowns, and mask	1,800	$1.26/hour × 1,800	2,268
Cost of universal influenza vaccination			
ICU HCWs	30	$10 × 30	300
HCWs, entire hospital	980	$10 × 980	9,800

*ICU, intensive care unit; RT-PCR, reverse transcriptase polymerase chain reaction; HCWs, healthcare workers.
†All 115 patients had acute phase serum samples tested for anti-H5 antibody; 42 (37%) were <60 years old and survived ≥14 days after hospitalization.
‡Estimated 1 min to put on and take off the protection gear with 100 encounters per day (*15*).

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Transplacental Chikungunya Virus Antibody Kinetics, Thailand

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Antibodies to chikungunya virus were detected by hemagglutination-inhibition assay in 33.6% of 2,000 infants' cord sera at delivery. Follow-up of 24 seropositive infants showed that the half-life of antibody persistence was 35.5 days. Chikungunya virus infection is common in Thailand, and routine use of diagnostic assays is needed.

Chikungunya virus (CHIKV, family *Togaviridae*, genus *Alphavirus*) was first isolated during an epidemic in Tanzania in 1952 and 1953 (*1*). CHIKV disease can manifest as a syndrome involving fever, rash, and arthralgia syndrome (*2*) and can produce clinical signs and symptoms that are difficult to distinguish from those of dengue fever or dengue hemorrhagic fever. CHIKV and dengue virus (DENV) are both transmitted by *Aedes* mosquitoes, such as *A. aegypti* and *A. albopictus*. Thus, many risk factors for CHIKV and DENV infections are similar. The diagnosis of dengue in Thailand is made primarily by clinical symptoms and a complete blood count according to World Health Organization guidelines. However, the major clinical features of dengue overlap with those of other causes of febrile illnesses (*3*). In addition, denguelike illness has occasionally been reported in patients without evidence of anti-dengue antibody seroconversion (*4*,*5*).

The objectives of this study were to assess the seroprevalence of antibodies to CHIKV in a sample of pregnant women and the kinetics of transplacentally transferred antibodies to CHIKV. This is the first study of serologic features of CHIKV in a large Thai sample. We also examined antibodies to dengue viruses in the same sample (*6*) to increase our understanding of the epidemiologic features of both diseases.

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The Study

Two thousand pregnant women with uncomplicated pregnancies at the time of delivery at the Phramongkutklao Hospital from March 1998 through October 1999 gave informed consent to participate in this study. Antibody titers to CHIKV were measured by hemagglutination-inhibition (HI) assay in all 2,000 cord serum samples. Antibodies in cord blood are transferred from the mother and can reflect previous infection. A subset of 250 mothers and their infants were enrolled to compare the rate of transfer of maternal antibodies. Within this subset, 101 infants had serial serum sampling at 1, 2, 4, 6, 9, 12, 15, and 18 months of age.

HI titers to CHIKV and DENV were determined at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Assays were performed according to the method of Clarke and Casals, modified for the microtiter system for each virus as previously described (*6*,*7*). HI titers ≥10 were considered positive. CHIKV is the only alphavirus known to circulate in Thailand; antibodies to other alphaviruses were not expected in this study, nor were they assayed. However, Ross River virus, Getah virus, Sindbis virus, and Bebaru virus have been reported to circulate in countries that border Thailand (*1*).

The mean age of the 2,000 mothers was 26.4 years (range 15–45 years). Most volunteers (79.9%) lived in Bangkok. Of these, 672 (33.6%) and 1,937 (96.9%) were seropositive for CHIKV and DENV, respectively. The seroprevalence of antibodies to CHIKV increased with age (Figure 1), and ≈47% of mothers ≥35 years of age were seropositive to CHIKV. The degree of CHIKV-specific antibodies transferred to infants was determined in 250 randomly selected mother-infant pairs. Of 250 mothers, 79 (31.6%) were seropositive for CHIKV, and 64 (81.0%) of these mothers transferred antibodies to their babies. We compared HI titers between mothers and cord sera; 58% had the same titers, 31% of cord sera had higher titers, and 11% of cord sera had lower titers. This finding was consistent with an active transport mechanism across the placenta. Similar findings were reported for DENV-specific antibodies (*6*,*8*). Fifteen (19%) infants born to seropositive mothers did not have detectable titers of antibodies to CHIKV.

Of the 79 mothers who were seropositive to CHIKV, 28 agreed to further follow-up study; their infants were followed up until 18 months of age. Four infants were negative on cord blood testing and remained negative until 18 months of age. Of 24 infants whose cord blood was positive, 8.3%, 33.3%, 87.5%, and 100% lost their antibodies to CHIKV by 2, 4, 6, and 9 months of age, respectively. The half-life of antibody to CHIKV was calculated by

[1]These authors contributed equally to this article.

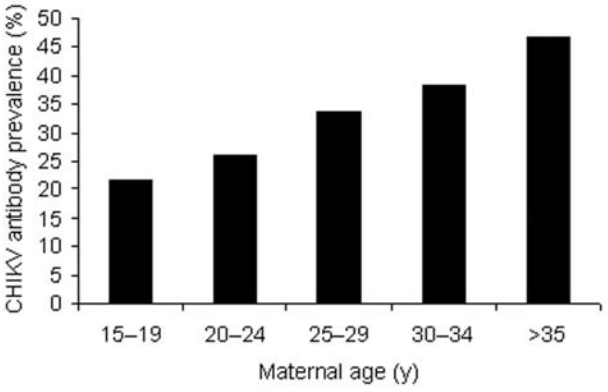

Figure 1. Age-specific seroprevalence of maternal antibody to chikungunya virus (CHIKV) measured by hemagglutination-inhibition assay in infant cord blood at the time of delivery.

plotting the antibody titer versus age to 18 months on both linear and logarithmic scales. Using SPSS software (SPSS Inc., Chicago, IL, USA), we calculated the line of best fit by exponential regression (Figure 2). From this curve, we calculated the half-life of maternal antibodies to CHIKV in infants to be 35.5 days.

Conclusions

In Thailand, after the first reported cases of CHIKV infection were confirmed by serologic analysis in 1960 (*9*), a serosurvey was conducted in 1976 in a rural population with an overall antibody prevalence of 24.6% that increased with age (*10*). The higher prevalence seen in this study (33.6%) might be explained by a larger sample that is more capable of sustaining transmission and increased vectors. A nationwide serologic study of both DENV and CHIKV in Thai patients took place in 1974–1976. This study suggested that CHIKV was not a substantial health problem (*11*). Disease caused by CHIKV, unlike DENV, is not a reportable disease, and laboratory diagnosis is not routinely available. As a result, it is probably underreported, which would reduce physicians' index of suspicion, resulting in further underreporting. However, epidemics of CHIKV disease in Thailand have been periodically documented.

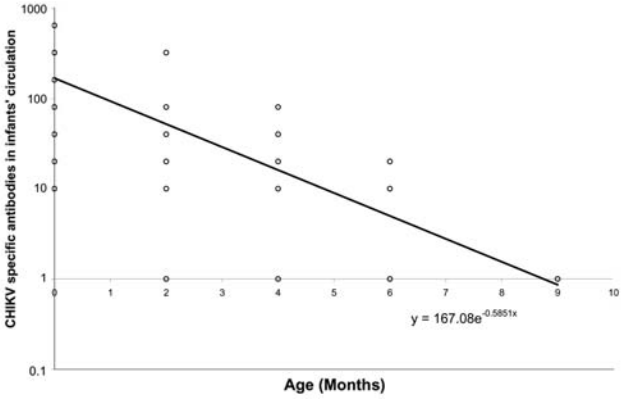

Figure 2. Half-life of maternal antibody to chikungunya virus (CHIKV). Each dot represents the titer at that given age; >1 participant can share the same dot. CHIKV hemagglutination-inhibition titers in infants' sera are plotted on a logarithmic scale, with the line of best fit calculated by exponential regression on a linear scale.

The clinical-to-subclinical ratio was 1:1 to 1:6 for DENV infection (*12*) and was ≈1.8:1 for CHIKV infection (C.G. Beckett, pers. comm.). This report shows the reemergence of CHIKV infection in Thailand; the ratio of seroprevalence of DENV infection to CHIKV infection was 2.9:1. National surveillance reported 130,000 dengue illnesses in 1998 (*13*). If the ratio is applied, we can estimate >44,000 persons infected with CHIKV in the same year. Therefore, infection with this virus may be more common than is believed. In a previous study of Thai children hospitalized with presumptive dengue hemorrhagic fever, ≈20% of diagnoses were ultimately changed to acute CHIKV infection (*4*).

Antibodies to CHIKV in infants' circulation may protect them from the illness until 9 months of age. This finding is consistent with the observation that denguelike illness is rarely seen in infants. A vaccine for CHIKV is still in early stages of development (*14*); the 9-month persistence of antibodies to CHIKV provides insight to the optimal age of vaccination, should a vaccine become available. This study shows substantial CHIKV circulation in a DENV-endemic country. Since signs and symptoms of the disease are similar, surveillance for viruses and the diseases they cause should be developed and maintained.

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etymologia

chikungunya

[chik′′ən-gun′yə]

From the language of the Makonde, northern Mozambique and southeast Tanzania (often misattributed to Swahili), "that which bends up." Chikungunya refers to the stooped posture that develops as a result of arthritic symptoms. A self-limiting disease, chikungunya is caused by an alphavirus spread by the bite of *Aedes* mosquitoes. Though not generally fatal, the disease can be severe. A widespread outbreak in islands of the Indian Ocean, begun in February 2005, has caused >200 deaths.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003 and wikipedia.org

Food Markets with Live Birds as Source of Avian Influenza

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A patient may have been infected with highly pathogenic avian influenza virus H5N1 in Guangzhou, People's Republic of China, at a food market that had live birds. Virus genes were detected in 1 of 79 wire cages for birds at 9 markets. One of 110 persons in the poultry business at markets had neutralizing antibody against H5N1.

Highly pathogenic avian influenza virus (HPAI) H5N1 infected 202 persons worldwide and killed 113 as of April 30, 2006 (*1*). Most patients were exposed to ill or dead birds or were involved in the slaughter or preparation of birds for human food (*2*). However, of 19 patients with confirmed cases in the People's Republic of China, 5 had no history of direct contact with ill or diseased birds and resided in urban or periurban areas that did not have farmed birds. We studied an infected patient from Guangzhou who did not report contact with birds.

The Study

The patient was from Guangzhou, the capital of the southern province of Guangdong. A fever (39°C) developed on February 22, 2006. He was hospitalized on February 26 and died on March 2. Diagnosis of influenza virus infection was made on March 3. Throat swab specimens obtained on March 1 and 2 tested positive for HPAI H5N1 virus by reverse transcription (RT)–PCR. Virus was isolated and named A/Guangzhou/1/2006 (H5N1).

Epidemiologic studies showed that the patient did not slaughter, process, or cook birds. However, while looking for work before his illness, he visited 9 food markets that had live birds. All 9 markets were located in the central part of the city (Table). He visited food market F twice a day from January 23 to 27 and food market G on February 17 for 30 minutes. Before his illness, he and his girlfriend (whom he lived with) shopped at markets B and F on February 20–22. He also visited food market I from February 10 to February 20. The dates he visited the other food markets could not be determined. Onset of fever occurred on February 22.

The food markets were typically large, clean, and well managed and had vendors selling vegetables, fruits, raw and cooked meats, food flavorings, beverages, and other goods. They are typical of larger food markets in cities in the People's Republic of China. The only difference between markets in Guanzhou in southern China and those in cities in northern China is that more (2–9) booths are used to sell live birds in Guanzhou. Wire cages are stacked next to each other with ≈5–10 birds in each cage (chickens, geese, ducks, and pigeons). Each species of bird is placed in separate cages; chickens are the most common species. All cages are located in a closed room separated by a glass window from customers, who choose the bird they prefer. When a live bird is selected, it is slaughtered in view of the customer. Sanitation inspections are routinely performed by municipal authorities. No diseased or dead birds were observed during this investigation.

Animal cages were swabbed and anal swabs of live birds were obtained at the food markets (Table) on March 3 and 4 and tested for HPAI by using RT-PCR (*3*) for the hemagglutinin (H5), neuraminidase (N1), and membrane (M) genes. Positive PCR results were confirmed by sequencing. None of 94 anal swabs from live birds tested positive for HPAI H5N1. However, 1 of 79 animal cage swabs tested positive for HPAI H5N1 (Figure 1). The positive swab was from a goose cage at market I (Table), the market that the patient visited from February 10 to February 20. The nucleotide sequences of H and M genes from specimens from this patient were compared with those from the animal cage swab and submitted to GenBank (accession nos. DQ842487–90). Forty-eight variations were found in the NA gene and 15 were found in the HA gene, which resulted in 17 HA amino acid and 3 NA amino acid changes, respectively. Phylogenetic analysis with the neighbor-joining method using the ClustalX program (*4*) suggested that the 2 strains are related to each other and to duck isolates (Figure 2).

Serum samples were obtained from 110 of 121 poultry purveyors working at the live bird food markets and screened for antibody to H5N1 to determine if subclinical infections occurred. One of 110 serum samples was positive (titer 320) by hemagglutination-inhibition assay with turkey erythrocytes (Lampire Biologic Laboratories, Pipersville, PA, USA) and H5N1 virus strains A/Hong Kong/486/97 and A/Vietnam/1194/04/H5N1 (*5*). Neutralizing antibody

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Table. Serum and swab sample results from live birds and animal cages sampled at markets in Guangzhou, People's Republic of China*

	Food market																			
	A		B		C		D		E		F		G		H		I			
Source	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S
Serum																				
Poultry purveyors	22	22	22	21	6	5	2	2	14	12	**14**	13	14	14	12	11	15	10	121	110
Swabs																				
Animal cages	20	10	27	0	8	8	4	0	16	3	28	8	27	18	24	8	27	**24**	181	79
Anal swabs																				
Chicken	160	10	190	6	64	6	36	5	95	6	268	7	195	3	160	1	205	13	1,373	57
Duck	10	0	5	1	4	1	0	0	0	0	3	1	3	3	2	1	10	5	37	12
Goose	5	0	0	0	0	0	0	0	3	1	3	1	0	0	3	1	6	4	20	7
Pigeon	15	0	20	3	10	2	0	0	0	0	0	0	15	2	10	1	70	5	140	13
Partridge	30	0	45	0	30	1	0	0	0	0	0	0	0	0	10	1	20	2	135	4
Quail	80	0	110	0	60	0	0	0	0	0	0	0	0	0	0	0	15	1	265	1

*T, total no.; S, no. sampled. Markets and samples from which specimens tested positive for virus genes or neutralizing antibody against highly pathogenic avian influenza virus H5N1 are in **boldface**.

titers against the 2 strains of virus were 1,280 and 640, respectively. The positive serum sample was from a 44-year-old man who slaughtered birds for 5 years. He slaughtered ≈100 chickens/day and did not report any recent respiratory diseases. He denied any contact with ill birds.

Conclusions

Our investigation suggests that the patient may have been infected by an unknown mechanism at a food market that had live birds. We detected H5N1 virus genes in a swab from a goose cage and neutralizing antibody against H5N1 in a poultry worker in 1 of the food markets the patient visited.

This case from Guangzhou was not an isolated event. Five patients with H5N1 infection with no history of exposure to diseased or dead birds before the onset of avian influenza have been reported in the People's Republic of China; 4 of these 5 patients visited markets that had live birds. The first patient was a 41-year-old woman from Fuzhou, the capital of Fujian Province, whose diagnosis was made in December 2005 (*6*). She visited a market that had live birds 2 weeks before her illness. Another patient lived in a periurban area of Chengdu, the capital of Sichuan Province; her diagnosis was made in January 2006. She was self-employed in a shop selling dry goods at a market that had live birds in Jinhua Town in Chengdu (*7*). Two other patients in urban areas were reported, 1 in Shanghai and 1 in Shenzhen. Influenza was diagnosed in the patient in Shanghai in March 2006, but this patient had no history of visiting a food market that had live birds or contact with diseased birds (*8*). Influenza was diagnosed in the patient in Shenzhen in April 2006; this patient reported visiting a food market that had live poultry before becoming infected with influenza virus.

Our findings suggest that food markets or farmers' markets that have live poultry may be a source for avian influenza infection in which healthy live birds may carry the virus. This was previously shown in Hanoi, Vietnam, in 2001, where H5N1 virus was detected in domestic birds in a live bird market (*9*). Serologic investigation also demonstrated low seroprevalence of antibody against HPAI H5N1 in poultry workers from this market. However, no outbreaks of HPAI among birds were reported until early 2004 (*10*). H5N1 virus may be sustained in poultry largely through the movement of poultry and poultry products, especially through domestic ducks (*11*,*12*). The introduction of H5N1 virus from healthy poultry (such as ducks) may be occurring where no outbreaks in healthy flocks

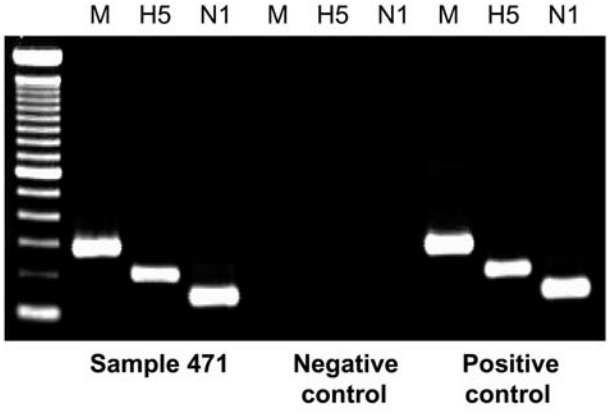

Figure 1. Detection of avian influenza virus H5N1 from an animal cage for geese by reverse transcription-PCR. Viral RNA was extracted from the sample and amplified by using 3 pairs of primers specific for membrane (M), hemagglutinin (H5), and neuraminidase (N1) virus genes. Sample buffer was used as a negative control, and viral RNA from a human H5N1 virus strain (A/Hong Kong/486/97) was included as a positive control. First lane, molecular mass ladder.

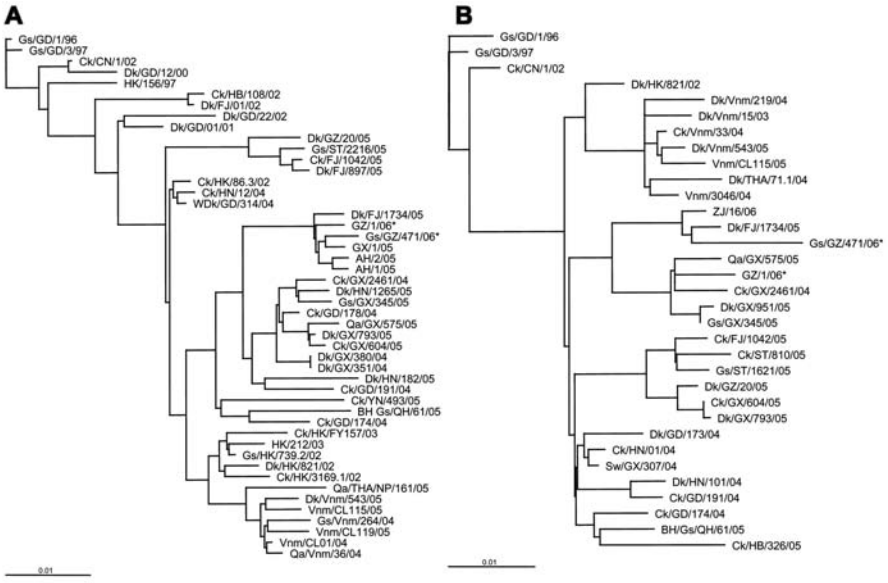

Figure 2. Phylogenetic relationships of representative H5N1 influenza virus strains and patient and animal cage isolates (indicated by asterisks) used in this study. A) Hemagglutinin gene (nt positions 29–1650). B) Neuramidase gene (nt positions 28–1323). Gs, goose; GD, Guangdong; Ck, chicken; CN, People's Republic of China; Dk, duck; HK, Hong Kong; HB, Hebei; FJ, Fujian; GZ, Guangzhou; ST, Shantou; HN, Hunan; WDK, wild duck; GX, Guangxi; AH, Anhui; Qa, quail; YN, Yunnan; BH Gs, brown-headed goose; QH, Qinghai; THA, Thailand, NP, Nakhon Pathom; Vnm, Vietnam; ZJ, Zhejiang; Sw, swine. Scale bars show percentage relatedness.

have been observed. Therefore, the virus is likely reintroduced at low levels and can infect persons visiting live poultry markets.

The cultural preference of eating freshly slaughtered birds is not unique to the People's Republic of China; it is also common in other Asian countries. Our results suggest that the practice of selling live birds directly to consumers in food markets should be discouraged in areas currently experiencing influenza outbreaks among birds, especially in large modern cities where there may be a threat to the casual market visitor (*2*,*13*,*14*).

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Recurrent Tuberculosis and Exogenous Reinfection, Shanghai, China

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Of 52 patients with recurrent tuberculosis in Shanghai, People's Republic of China, 32 (61.5%) had isolates in which genotype patterns of *Mycobacterium tuberculosis* differed between first and second episodes. This result indicates that exogenous reinfection is common in an area with a high incidence of tuberculosis.

Elucidating the role of reinfection in tuberculosis (TB) recurrence is important in the People's Republic of China because this country has the second highest incidence of TB in the world, an estimated rate in 2004 of 101 cases/100,000 persons/year (*1*).After effective short-course therapy for active TB, some patients experience another, recurrent TB episode. The recurrent episode may be due to endogenous reactivation or exogenous reinfection. The role of exogenous reinfection has been debated for decades (*2*,*3*). Understanding the cause for recurrence helps clinicians evaluate the effectiveness of therapeutic regimens and TB prevention and control programs to assess strategies and interventions.

DNA fingerprinting techniques provide excellent tools to address whether recurrent TB is caused by endogenous reactivation or exogenous reinfection. Different *Mycobacterium tuberculosis* strains can be differentiated by genotyping methods that use information about genetic markers and their distribution in the genome (*4*). Among persons with recurrent TB, if the isolates from 2 TB episodes have the same genotype, the episode is defined as an endogenous relapse; otherwise, it is defined as exogenous reinfection. Previously, researchers have tried to assess the relative importance of endogenous relapse versus exogenous reinfection, with varied results (*2*,*5*–*10*). Our study helps elucidate the role of reinfection in TB recurrence in China.

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The Study

Shanghai is 1 area in China with high TB treatment success rates. Persons with TB symptoms (mainly cough for at least 2 weeks, chest pain, weight loss, and fever) can go to any hospital or community health center in Shanghai. They are first screened by chest radiograph. All patients with suspected TB are referred to a TB hospital, where sputum is examined by smear and culture. TB is bacteriologically confirmed if $\geq$1 sputum smear examination result was positive for acid-fast bacilli or if the culture was positive. The TB hospital sends all mycobacteria-positive cultures to the TB reference laboratory at the Shanghai Municipal Centers for Disease Control and Prevention (Shanghai CDC), which participated in the World Health Organization/International Union against Tuberculosis and Lung Disease global drug resistance surveillance project, for species identification and drug susceptibility testing. TB patients are treated in the TB hospital during the intensive phase. On the basis of the sputum smear and culture examination 1 or 2 months after TB therapy is initiated, the patient is discharged from the hospital and finishes treatment as an outpatient. The community health center physician trains family members to supervise and observe the TB patient's remaining doses and treatment. Completion of anti-TB therapy is based on the examination of sputum smear, culture, and chest radiographic results. Shanghai CDC collects and manages patient information, such as social and demographic characteristics, clinical data, TB treatment regimens, and the result of drug susceptibility testing and species identification.

From January 1999 through September 2004, Shanghai CDC collected 6,442 clinical isolates from a total of 6,960 persons with bacteriologically confirmed (by smear or culture) TB. Of these case-patients, 5,688 were cured, and 202 (164 male and 38 female) had a recurrence, defined by the following criteria: 1) their TB episode was confirmed by culture; and 2) they experienced 2 successive TB episodes, with cure as the outcome of the first episode. Cure was defined as the completion of a standard course of combination therapy and successive negative sputum cultures during treatment. At the same time, chest radiography showed resolution of the focus of infection. On the basis of the selection criteria, 54 patients with recurrent TB were included in the study (Figure 1).

The mycobacterial interspersed repetitive unit (MIRU) typing method (*11*) was used to genotype strains from these patients. This method is relatively easier to perform and less technically demanding than IS*6110* restriction fragment length polymorphism (IS*6110*-RFLP), which was used in many previous molecular epidemiologic studies of TB (*12*). We followed the protocol described by Kwara et al. (*13*) with modifications. PCR products were analyzed by 2.5% (w/v) agarose gel electrophoresis

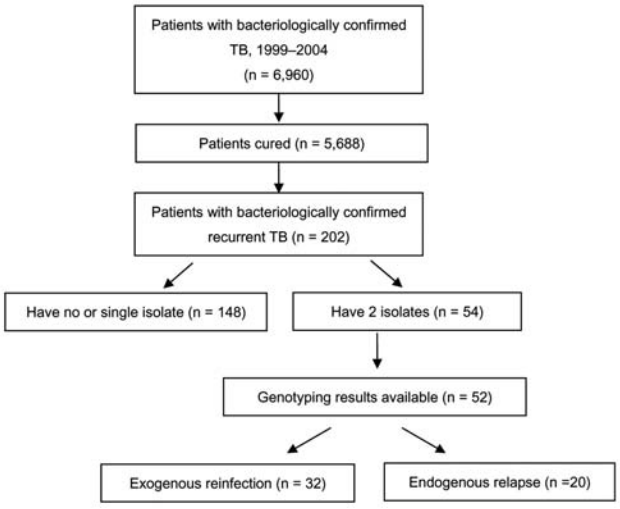

Figure 1. Selection of patients in the study, Shanghai, People's Republic of China, 1999–2004. TB, tuberculosis. The total number of patients from 1999 through 2004 was 6,960; among these patients, 5,688 were cured.

(Figure 2A). Genotyping was performed for 2 isolates. We analyzed the data for the remaining 52 patients and found MIRU patterns for both episodes to be the same for 20 patients and different for 32. Of these 32 patients, 13 had 1 MIRU locus change between the 2 isolates, 10 had a change in 2 loci, and 9 had ≥3 loci changes in their isolates (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no11/05-1207_appT.htm). These results indicate that 32 (61.5%) of 52 (95% confidence interval 47.0%–74.4%) of the recurrent cases were due to reinfection. To further validate the MIRU genotype result, the IS*6110* RFLP genotyping method was performed; results showed that the isolates with 1 or 2 MIRU locus changes had very different RFLP patterns (a difference in ≥4 bands in the IS*6110*-RFLP, Figure 2B).

We further used patient age group and intervals between the 2 episodes to classify recurrent TB. We found that the percentage of TB patients with an exogenous reinfection decreased with age from 100% (TB patients <30 years of age) to 66.7% (TB patients 30–60 years) and 53.3% (TB patients >60 years). We also found that the frequency of exogenous reinfection increased with the amount of time that elapsed between the end of TB treatment for the first episode of TB and the date that the second episode was diagnosed. Exogenous reinfection accounted for 7 (46.7%) of the 15 recurrent episodes that occurred within 6 months after treatment for the first episode; the percentage of recurrent cases due to exogenous reinfection increased to 73.9% (17/23) among TB patients whose second episode occurred ≥1 year after treatment for the first episode.

Conclusions

We analyzed genotypes of 104 isolates from 52 patients who experienced 2 TB episodes from 1999 through 2004. Thirty-two of 52 patients had different MIRU genotype patterns in clinical isolates from their 2 episodes, which indicates that exogenous reinfection accounted for 61.5% of the recurrent cases in Shanghai during the study period. The high proportion of exogenous reinfection in recurrent TB patients indicates that high levels of transmission of *M. tuberculosis* are an important cause of TB in Shanghai, China.

Although several reports have indicated that exogenous reinfection may occur after successful treatment, the proportion of TB cases that are actually caused by exogenous reinfection may vary dramatically for several reasons, such as the patients' HIV infection status, different facilities, and healthcare providers' various definitions (some used different numbers of days elapsed between the first and second episode to define a recurrent case of TB), and particularly the small sample size (*2*). Several studies have reported that HIV may be a risk factor for exogenous reinfection (*2*,*14*,*15*). Unfortunately, we do not have data on each patient's HIV infection status, and we cannot totally exclude the effect of HIV infection. However, considering the low incidence of HIV infection among residents of Shanghai (≈0.6 cases/100,000 persons/year), we consider it unlikely that HIV is a major factor in our findings. The criteria used to define recurrent TB cases differ; various

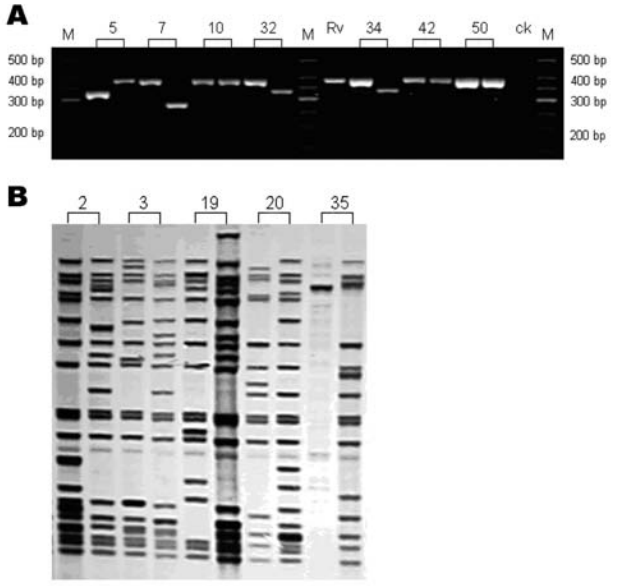

Figure 2. Genotyping analysis of clinical isolates from patients with recurrent tuberculosis. Numbers represented the patients' codes. A) Gel electrophoresis analysis of the PCR products of the mycobacterial interspersed repetitive unit (MIRU) locus 10. bp, base pair; M: DNA marker; Rv, H37Rv positive control; ck, negative control. B) IS6110 restriction fragment length polymorphism analysis of some patients with different MIRU patterns.

studies defined the interval between the end of TB treatment and a new episode (recurrent TB) as 3–12 months (*5*,*7*,*8*). Our study did not define the interval and determined that 61.5% of the recurrent TB cases were due to exogenous reinfection. If we chose a 6-month interval to define a recurrent TB case, then we would find an even higher percentage of recurrent TB cases were due to exogenous reinfection (67.6%, 25/37).

Previous studies and our study demonstrate that TB patients can be reinfected with a new strain of *M. tuberculosis* after treatment, which indicates that the immunity evoked by the primary infection does not protect the patient against a later infection. A recent study from South Africa demonstrated that the rate of TB reinfection after successful treatment is even higher than the rate of new TB infection (*5*). Such results suggest major challenges for the development of a new vaccine that will be effective against *M. tuberculosis*.

In summary, our study showed that 61.5% of recurrent TB cases in Shanghai from 1999 through 2004 were due to exogenous reinfection and confirmed that reinfection may be common in areas with a relatively high incidence of TB. This finding provides important implications for TB control. To prevent recurrent TB, more attention should be paid to the interruption of TB transmission.

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Identical Genotype B3 Sequences from Measles Patients in 4 Countries, 2005

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Surveillance of measles virus detected an epidemiologic link between a refugee from Kenya and a Dutch tourist in New Jersey, USA. Identical genotype B3 sequences from patients with contemporaneous cases in the United States, Canada, and Mexico in November and December 2005 indicate that Kenya was likely to have been the common source of virus.

Identification of measles virus genotypes is a valuable tool for epidemiologic investigations and evaluation of control activities in countries that have eliminated indigenous measles. Many of the 23 recognized genotypes of measles are associated with countries or regions with endemic measles (*1*). Measles genotypes in clade B (genotypes B1, B2, B3) are associated with endemic circulation of measles in various countries in sub-Saharan Africa (*2*). The prototype clade B viruses were isolated in 1983 in Cameroon (B1) and in 1984 in Gabon (B2). Hanses et al. (*3*) proposed a new genotype, B3, after characterization of several viruses collected in 1997 and 1998 in Ghana and Nigeria. Sequencing studies of additional viruses from Africa demonstrated that the proposed subdivision of the B3 viruses into subgroups B3.1 and B3.2 was epidemiologically useful for describing 2 distinct clusters of contemporary B3 viruses (*4*,*5*).

Because measles is highly infectious, international travel originating from measles-endemic areas can result in sporadic cases of measles in countries that have eliminated indigenous transmission. International visitors may infect other travelers while moving through transportation hubs or tourist areas; such cases would not be detected unless the traveler sought medical attention or additional cases were detected. Thus, in many of these instances, the source of virus is unknown. We describe the contribution of global surveillance for measles virus genotypes in identifying a common source of virus among contemporaneous cases identified in the United States, Canada, Mexico, and the Netherlands.

The Study

On November 9, 2005, a 17-year-old man who arrived at the airport in Newark, New Jersey, United States, had symptoms consistent with measles. The man was part of a group of 148 refugees from the Eastleigh community in Nairobi, Kenya, who arrived in the United States from November 3 through 15. Genotype B3 (subgroup B3.1) was identified from virus samples from this patient; the sequence was identical to sequences from measles viruses collected in Nairobi and Machakos, Kenya, in October 2005 (Figure). All but 1 of the 6 viruses collected from Nairobi (Figure, MVi/Nairobi.KEN.xx.05) were from patients from the Eastleigh area of Nairobi, where an outbreak of measles had been reported in the Somali and Ethiopian communities (*6*).

Also in November 2005, a single case of measles was reported in the Netherlands. This patient had visited New York City, returned to the Netherlands on November 15, noted a rash on November 23, and was hospitalized with pneumonia and fever on November 24. The initial investigation focused on potential settings where exposure may have occurred in New York City. The source of infection was traced to an unrecognized exposure to the patient in New Jersey only after analysis of the Netherlands viral sequence demonstrated complete identity with the New

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Jersey genotype B3 virus. The possibility of an epidemiologic link between the 2 cases led to the discovery that the Dutch visitor had arrived at the Newark airport on November 9 and waited in the arrival area for 1 h, along with the group of refugees from Nairobi.

Subsequently, genotype B3 was identified from patients who had had measles during December 2005 in Texas, Canada, and Mexico. In Texas, during the first 2 weeks of December, 3 cases of measles were reported in members of a family from Houston. The patients had flown directly from Houston to the resort area of Cabo San Lucas, Mexico, where they stayed from November 22 through 27. In Mexico, health authorities reported 5 cases of measles beginning on December 12 among baggage handlers and other airport workers at the Mexico City airport. In New Brunswick, Canada, a patient developed a rash on December 19.

Although the earlier cases in New Jersey and in the Netherlands could be traced directly to the outbreak in Kenya, the sources of the cases in Texas, Canada, and Mexico were unknown. However, the sequences from Texas, Canada, and Mexico were identical to the sequences directly linked to the outbreak in Kenya (Figure). Measles viruses in the same chain of transmission have identical sequences (*7*,*8*), which indicates that the source of the virus for the cases in Texas, Mexico, and Canada was likely to have been the outbreak in Kenya.

Virus transmission may have occurred through contact with international travelers in airports or during transit because epidemiologic investigations did not detect other measles cases in Cabo San Lucas or Texas. The exception to possible air travel–related exposure was the single case that occurred in Canada. This patient had traveled by car, although investigations found no measles cases in the areas visited: Bangor, Maine (December 2); Boston, Massachusetts (December 3–6); and Portsmouth, New Hampshire (December 6). Two refugees from Eastleigh settled in a state visited by the Canadian patient. They entered Massachusetts on November 10, 2005, and by 21 days after arrival, measles had not developed. However, a measles outbreak was detected in southern Germany in January 2006 (*9*), and the viral sequence matched that of the Kenya outbreak virus (Figure; MVs/Stuttgart.DEU/4.06), which indicates that the source of this outbreak was also likely to have been Kenya. Therefore, B3 viruses with identical sequences could have been introduced into Texas, Mexico, or Canada by travelers infected with B3 virus in Europe.

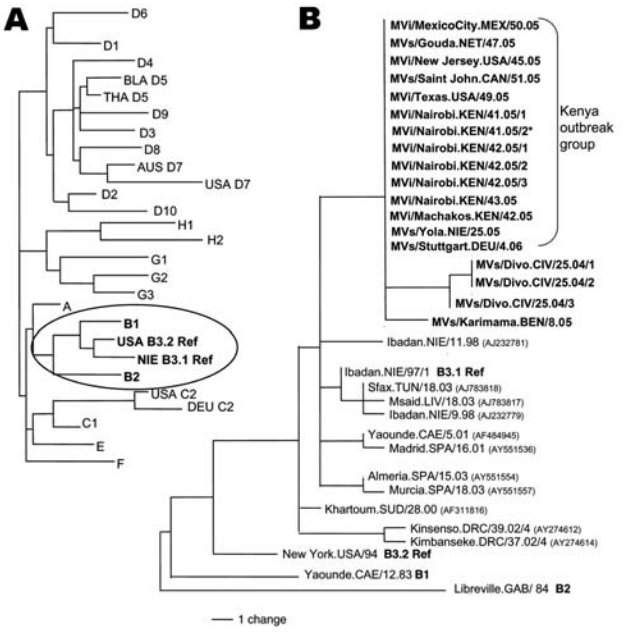

Figure. A) Dendrogram showing the relationships among the measles reference strains representing the 23 known measles genotypes (B3 has 2 reference strains). Clade B (circled) is expanded in panel B. B) Midpoint-rooted maximum parsimony tree of nucleoprotein genes (450 nt) of measles viruses from patients in the United States, Mexico, the Netherlands, Canada, and Kenya during 2005 and 2006. The unrooted tree includes sequences from Nigeria in 2005, Germany in 2006, Côte d'Ivoire in 2004, and Benin in 2005 (sequences in bold, this study) as well as selected B3 sequences available from GenBank for comparison. GenBank accession numbers are shown in parentheses. The identical sequences from the "Kenya Outbreak Group" are represented by Genbank accession number DQ888751, MVi/New Jersey.USA/45.05. The GenBank numbers for the sequence from Benin (BEN) and the 3 sequences from Côte d'Ivoire (CIV) are EF031461, EF031458, EF031459, and EF031460. *Collected from the Dagoretti area of Nairobi; the other Nairobi sequences were from cases in the Eastleigh area.

Conclusions

Although genotype B3 has been the most frequently detected measles genotype in western and central Africa (*4*, *10*–*12*), ours is the first report of the detection of genotype B3 in Kenya. Moreover, the sequence from a virus isolated in Nigeria in June 2005 (Figure; MVs/Yola.NIE/25.05) was identical to the sequences in the Kenya outbreak group. Although a link has not been established between Nigeria and Kenya, a survey of measles genotypes in Kenya in 2002 detected only genotype D4 viruses (*13*). Sequences of viruses isolated during 2004 and 2005 from Côte d'Ivoire and Benin (this study) were included in our analysis (Figure) because these B3 sequences represent closely related viruses from western Africa.

The analysis and dissemination of viral sequences from measles cases led to the identification of an unrecognized epidemiologic link at an airport and linked sporadic cases in 4 countries that do not have endemic measles to an ongoing outbreak in Kenya. Investigators in the field need

to collect adequate specimens for virus isolation. Timely communication of sequence data among epidemiologists and microbiologists is critical for identifying possible links among sporadic cases of measles. The potential for rapid transmission of measles during brief encounters with international travelers underscores the importance of global surveillance of measles virus.

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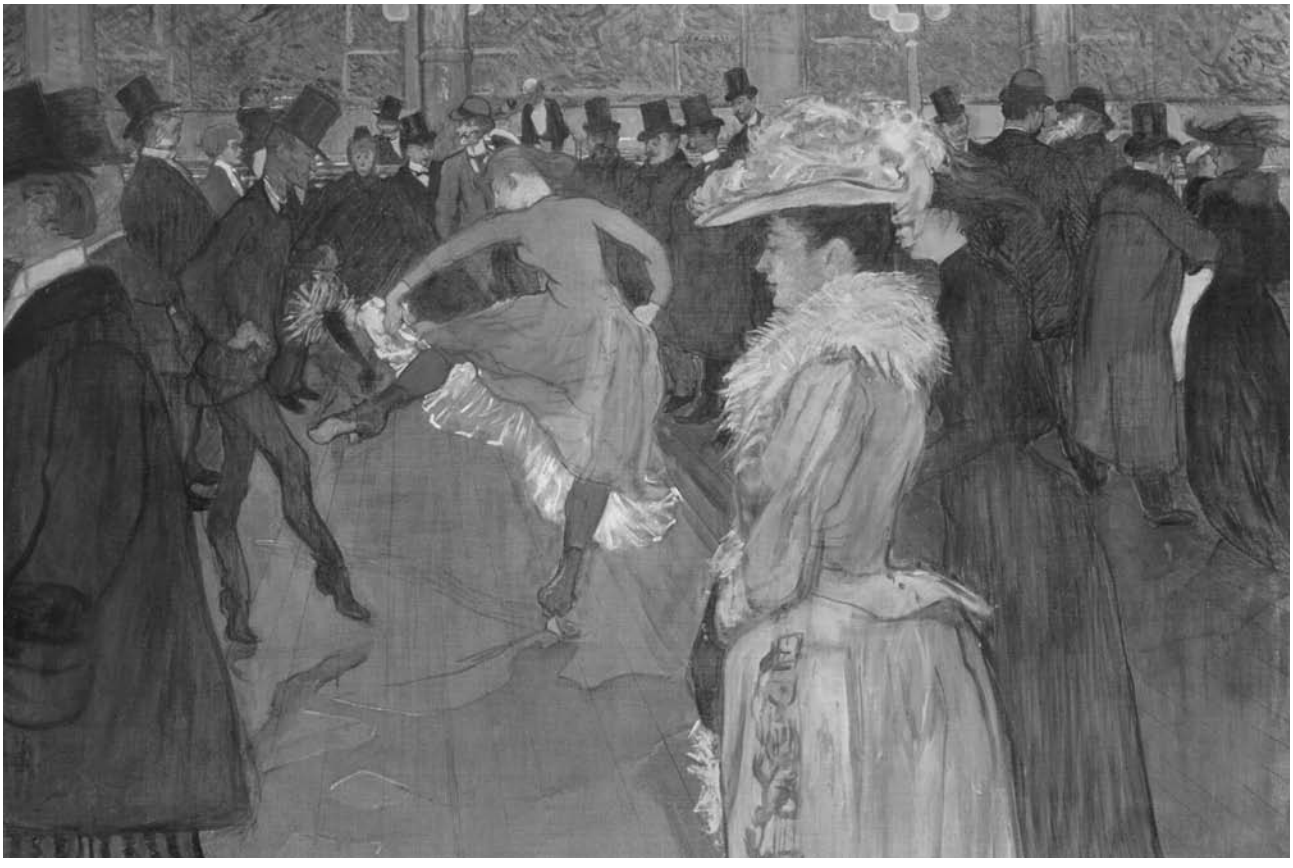

Henri de Toulouse-Latrec (1864–1901). At the Moulin Rouge: The Dance (1890). Oil on canvas (115.6 cm × 149.9 cm). The Henry P. McIlhenny Collection in memory of Frances P. McIlhenny, 1986. Philadelphia Museum of Art, Philadelphia, Pennsylvania, USA.

Sexual Health in Art and Science

Salaam Semaan,* Don C. Des Jarlais,† and Steve Bice‡

Artists and scientists express their understanding of sexual behavior differently. Artists use visual and spatial composition; scientists use collection, analysis, and interpretation of data. However, both art and science are testaments to the creative ability of the human mind.

Scholarly work that combines art and science is often delightful. Many biomedical journals, including the Journal of the American Medical Association, Clinical Infectious Diseases, and Emerging Infectious Diseases, display images of art objects, and some relate art to health (*1*) to put a human face on the technical content. For the most part, sexual health texts use graphic illustrations to show clinical manifestations of infection and disease. Can fine art also be used to discuss sexual health?

In this article, we examine 6 art objects from the Philadelphia Museum of Art in the context of sexual health, especially the prevention and control of sexually transmitted diseases (STDs), including HIV (*2*). We combine 2 traditional approaches in our discussion of these 19th- and 20th- century pieces: chronology and theme (sexual health).

We begin with At the Moulin Rouge: The Dance (1890) by Henri de Toulouse-Lautrec (1864–1901). Although this artist was born to an aristocratic French family, he preferred the company of bohemians. As a teenager, Toulouse-Lautrec fell twice, injuring both legs. His

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stunted growth was attributed to those injuries; however, more recently, doctors have blamed a rare genetic abnormality associated with dwarfism (*3*). He reached maturity with a body trunk of average size but abnormally short legs. Despite these physical limitations, he found comfort among the vivacious crowds of Paris nightclubs and brothels (*2*). Toulouse-Lautrec frequented the Moulin Rouge, a fashionable night club in the Montmartre section of Paris. Its clientele included members of the upper class, sex workers, foreign tourists, and provincial rustics (*2*).

At the Moulin Rouge: The Dance portrays a mix of opposites: bright and dull colors, active dancers and passive spectators, merriment and monotonous leisure. Two dancers move energetically in the center of the canvas. The female dancer raises her skirt as she kicks out her red-stockinged legs. She gyrates so vigorously that her chignon has fallen and her skirt flares out. Her partner in a top hat is standing on tiptoe, kicking his feet as they engage in what was considered a crude, sexual dance (*4*). Surrounding the dancers is a crowd: men in top hats, a woman in a bright pink dress, other dancers, customers at the bar. Judging from her ostentatious attire and feathery hat, people at that time might have identified the woman in the pink dress as a sex worker (*2*).

In this painting are scandalous subjects: a crude dance and a sex worker (*4*). Then, as today, negative attitudes prevailed toward sex workers, who were seen as carriers of STDs (*5*,*6*). Although Toulouse-Lautrec greatly admired Edgar Degas, Degas took only passing notice of Toulouse-Lautrec, saying that some of Toulouse-Lautrec's studies of women "stank of syphilis" (*4*), which at that time was as feared as HIV/AIDS today. In Europe in the 19th century, more than 15% of the adult population and 70% of sex workers were estimated to have been infected with syphilis (*7*,*8*). Today, as then, sex workers may be viewed as immoral carriers of physical and moral hazards, including HIV/AIDS and other STDs. In turn, sex workers may mistrust healthcare providers and public health practitioners and, as a result, may not notice health messages and treatment services. Male and female sex workers may be victims of their social and economic environment, driven to sex work by poverty and lack of educational and job opportunities.

Despite his aristocratic upbringing, Toulouse-Lautrec found a way to accept and feel accepted by the entertainment industry (*4*). Sex workers were his friends, and he treated them as equals (*4*). Similarly, some public health practitioners may view sex workers as positive agents for sexual health and engage them in screening, preventive, and curative interventions for STD control and prevention.

Until the advent of penicillin in 1943, treatment for syphilis was based on the use of heavy metals such as mercury (*9*) or, as the saying goes, "a night in the arms of Venus leads to a lifetime on Mercury" (*10*). In the late 1980s, we learned that concurrent HIV infection can turn secondary syphilis back to the serious illness it was before penicillin (*11*); however, HIV-infected patients can be treated for syphilis with penicillin (*12*). At the time of Toulouse-Lautrec, who may well have contracted syphilis from 1 of his models, penicillin was not available (*4*). Shortly before his death, Toulouse-Lautrec entered a sanatorium, probably because of the adverse effects of tertiary syphilis. He died of alcoholism and syphilis at age 36 (*4*). Toulouse-Lautrec's sympathetic depiction of cabaret dancers humanized his era's sex workers. Can today's public health establishment improve the lives of HIV- and STD-infected sex workers? Today, many public health practitioners counsel sex workers about preventive and treatment services to protect them and their clients and provide them with the prospect of health and safety. Sex workers can also receive social and economic opportunities to enable them to leave the sex work industry.

Pierre Auguste Renoir (1841–1919). The Great Bathers (1884-1887). Oil on canvas (117.8 cm × 170.8 cm) The Mr and Mrs Carroll S. Tyson, Jr Collection, 1963. Philadelphia Museum of Art, Philadelphia, Pennsylvania, USA.

While Toulouse-Lautrec painted the entertainment world, another French artist, Pierre-Auguste Renoir (1841–1919), portrayed the sensuous side of women. His paintings celebrated fresh air, dazzling sunlight, and pleasures of the senses (*13*). In The Great Bathers (1884–1887), Renoir shows 5 nude women bathing. Two lounge beneath a tree on a verdant riverbank while a third teasingly threatens to splash 1 of them. Further away, 2 other women frolic, seemingly indifferent to anything but the play of the hot sun and the cool fresh water on their bodies. Renoir blesses his women with luminous skin and uses color to suggest roundness. He paints them precisely, with a clean line surrounding their contours, portraying their beauty and love of life. To separate the women from the landscape, Renoir uses lemon yellows and lavenders, which

Paul Cézanne (1839-1906). The Large Bathers (1906). Oil on canvas (210.5 cm × 250.8 cm). Purchased with the W. P. Wistach Fund, 1937. Philadelphia Museum of Art, Philadelphia, Pennsylvania, USA.

create an airy image of the landscape. Translucent, bright summer light flickers on the trees and glistens on the water.

Apparently, Renoir did not see an ugly side to nudity; the woman in the middle of the painting was Madame Renoir. Looking at this painting, one wonders how people would behave if moral codes were not enforced and sexual infections did not exist. How did HIV/AIDS and other STDs originate? These infections take away from the pleasure of sex. In the late 1990s, it was shown that HIV-1, a retrovirus of animal origin, had probably originated from the *Pan troglodytes* species of chimpanzees, in which the virus coevolved over centuries (*14*). Because chimpanzees were killed for food in parts of sub-Saharan Africa, the species jump probably occurred when a hunter was exposed to the blood of an infected chimpanzee during its butchering. After the accidental transmission of the virus to humans, from infected primates and probable genetic mutations, HIV spread rapidly among population groups, facilitated by changes in global social and economic conditions (*15*).

At the time Renoir painted The Great Bathers, syphilis was prevalent and HIV epidemics did not exist (*16*). By the end of 2005, an estimated 40.3 million people worldwide were living with HIV and more than 25 million had died of AIDS (*17*). Almost 14,000 persons worldwide become infected with HIV each day, and 5 million become infected each year (*17*). The development of a safe and effective vaccine for HIV remains a formidable challenge (*16*), so safe sex is critical for disease prevention and control. As one looks at the frolicking bathers, one can vicariously enjoy their merriment and contemplate STD prevention and control, which supports human capacity for sexual intimacy within healthy relationships.

Renoir was not the only painter of nudes. Paul Cezanne (1839–1906), also a French artist, painted The Large Bathers in 1906. This work portrays 14 figures with obliterated faces and truncated limbs. The ambiguity of the bathers' sex may stem from the fact that Cezanne did not use live models. He made sketches based on paintings and sculptures in museums that he later transposed to canvas.

The 14 figures in the foreground of The Large Bathers are clustered in 2 groups, each forming a small pyramid, on each side of the painting. The figures are in an airy setting defined by refracted light and tall, slanting trees that form a pointed arch above them. Behind the figures is a person swimming. On the opposite shore appear 2 more figures. Cypress trees and a church steeple emerge from the distant wooded landscape. With somber blues, greens, and ochers, Cezanne integrates the figures into their surroundings.

How can this group of nude figures be related to sexual health? Although traditionally, individual sexual behavior has been analyzed as a determinant of HIV/AIDS and STDs, more recently, sexual mixing and sexual networks have been recognized as important mechanisms for explaining population and racial disparities in infection rates (*18*). Demographic and environmental factors create social and sexual networks that influence population-level variations in sexual behavior and infection rates of STDs and HIV. Arguably, the 14 figures could form a large sexually active group, a potentially at-risk pool for transmission of HIV/AIDS and other STDs. Public health interventions try to change peer and community norms regarding sexual health (*19*).

Concurrent sexual partnerships also explain generalized heterosexual HIV/AIDS epidemics (*20*). Serial monogamy and sporadic sexual encounters might not contribute as much to new infections as do networks of longer term concurrent or overlapping partnerships. If, for example, 1 person in a network characterized by concurrent partnerships is infected with HIV, everyone is at high risk because more people are exposed to the virus and because recently infected persons have manyfold higher viral loads and are more infectious (*21*).

Toulouse-Lautrec's painting of an entertainment hall and Renoir's and Cezanne's paintings of nude bathers show how 19th- century male artists had the liberty to congregate in unconventional venues and to paint nude figures. Contemporaneous female artists often chose more socially acceptable themes, as shown by the Maternal Kiss (1897), by Mary Stevenson Cassatt (1844–1926) (*22*). This American artist left Philadelphia to study art in Paris in 1866. Because the Ecole des Beaux-Arts did not admit women, she studied with individual artists and was drawn

to the group derisively called the "impressionists" (*23*). Like Renoir, Cassatt became known as a portrait painter. She focused almost exclusively on the depiction of mothers and children.

Maternal Kiss portrays an intimate and tender moment between a mother and child. The child forms the psychological focus of the painting as the mother's features are lost in the child's cheek. The painting displays a subtle richness of color in the iridescent salmon-hued leg-of-mutton sleeve of the mother's dress and the delicate fabric clothing of the auburn-haired baby.

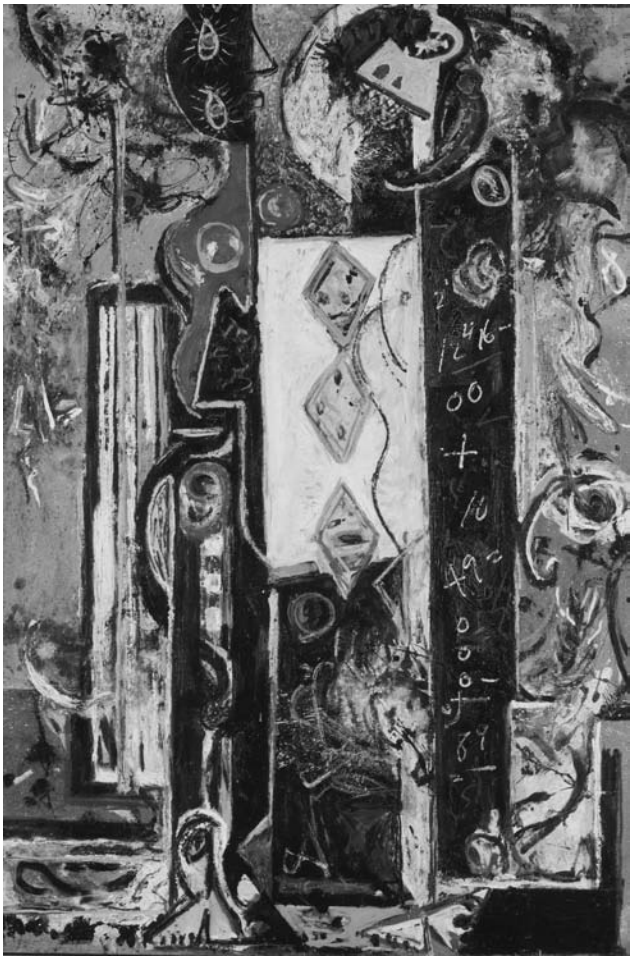
In an ideal world, mothers pass on to their children their love and wisdom; however, mothers infected with HIV or other viral STDs can pass these infections to their babies (*24*,*25*). An infant can acquire HIV infection during pregnancy, labor, delivery, or breastfeeding (*24*). Although perinatal HIV infections in the United States peaked in 1991 at an estimated 1,650, they declined in 2002 to an estimated 144–236 (*24*). Preventive and curative interventions have reduced perinatal HIV transmission in the United States to less than 2%, compared with 25%–30% without such interventions (*26*). Effective interventions include routine HIV screening of pregnant women, use of antiretroviral drugs for treatment and prophylaxis, avoidance of breastfeeding when the mother is HIV-infected, and use of elective cesarean delivery when appropriate. However, approximately 1,800 HIV-infected infants are born each day worldwide, most of them in sub-Saharan Africa (*27*). In 2003, an estimated 700,000 new HIV infections occurred in children worldwide—almost all from mother-to-child transmission.

At the time of Cassatt, congenital syphilis was a major concern because it caused miscarriages and stillbirths (*28*). A tragic possibility is that even today, a baby could be spared HIV infection, only to a die a few weeks later of congenital syphilis (*29*), as has been reported recently in several countries (*30*,*31*).

Marcel Duchamp (1887–1968), an American artist, born in France, was a scion of an artistic family. His Given (also known as Etant Donnes: 1. la chute d'eau, 2. le gaz d'eclairage [Given: 1. The Waterfall, 2. The Illuminating Gas])(image not shown) is a unique example of an art installation and presents a complex narrative in a multimedia format. Given (1946–1966) shows a naked woman behind a closed door. In the center of roughly stuccoed wall is a large arched doorway made of old bricks (*32*). The door is weathered silver gray, studded with iron rivets, and shows no sign of hinges, knob, or handle, confirming the impression that the door cannot be opened. In the middle of the door, at eye level, 2 small holes invite inspection of the 3-dimensional tableau that lies behind. As the viewer steps onto a mat in front of the door, the lights become activated so the viewer can peer through the holes for a private experience of what is within. Gazing through several layers of space, the viewer sees a nude woman lying on her back among a mass of twigs and leaves. Her face is farthest away and hidden by a wave of blonde hair. Her legs are spread and extend toward the door; her feet are obscured by the brick wall. Her right arm cannot be seen, but her left arm is raised, holding in her hand the glass fixture of a small gas lamp that glows faintly. In the distance is a hilly, wooded landscape that rises above a pond. Clouds are soft and white in the blue sky. To the far right is a waterfall.

Mary Cassatt (1844–1926). Mother and Child (Maternal Kiss) (1897). Pastel on paper (55.9 cm × 45.7 cm). Bequest of Anne Hinchman, 1952. Philadelphia Museum of Art, Philadelphia, Pennsylvania, USA.

Duchamp's installation can be disturbing, as one is suddenly confronted with an unexpected and shockingly graphic image of a naked woman behind the door. In this installation, Duchamp has determined forever the exact amount of detail and the fixed perspective he intended for the viewer. One is unable to walk around Given, to get closer to peer at details, or to back away for a different perspective. Similarly, talking about sex, even in the context of prevention and control of infection, can be disturbing. Because sex is a private matter, to optimize prevention and treatment, scientists and healthcare providers depend on the information provided by research participants and patients. Accurate reporting is crucial for treating patients and their sex partners, for monitoring trends of sexual



Jackson Pollock (1912–1956). Male and Female (1942). Oil on canvas (186.1 cm × 124.3 cm). Gift of Mr and Mrs H. Gates Lloyd, 1974. Philadelphia Museum of Art, Philadelphia, Pennsylvania, USA. Copyright 2006 The Pollock-Krasner Foundation/Artists Rights Society (ARS), New York, New York, USA.

behavior and infection rates, and for prevention and treatment. Inaccurate reporting can distort clinical decisions; can compromise diagnostic, preventive, and therapeutic interventions; and can hinder partner notification and referral services. Therefore, public health practitioners and healthcare providers strive for rapport and trust with research participants and patients.

Because sexual behavior is influenced by personal and societal attitudes, reporting and sharing of information is often subject to reporting bias, which arises when people do not reveal private information, even for health reasons. This type of bias is referred to as "social desirability bias" because what is considered socially desirable or undesirable behavior affects whether a person reports it accurately. Studies show that reporting of sexual behavior and infection status can be inaccurate, even when such information is shared with healthcare providers (*33*). Just as Duchamp challenges and disturbs the viewer, patients often challenge public health practitioners and healthcare providers. Successful public health interventions must overcome the uncomfortable aspects of sexual health communication.

The validity of reported data about sexual health can be enhanced. Procedures and laws protect people's privacy and the confidentiality and security of collected data (*34*). To maximize self-report accuracy, investigators ask respondents to provide information on recent sexual behavior with short recall times, e.g., "over the past 4 weeks" rather than "over the past 2 years" (*35*). Investigators also administer questionnaires in a confidential manner. They use self-administered questionnaires and computer-assisted technology, such as audio or telephone computer-assisted self-interviewing (*36*,*37*). Biologic markers are often used to ascertain validity of reported data (*38*).

With Duchamp's Given, we noted the importance of accurate reporting of sexual behavior. To note the importance of communication between partners, we explored Jackson Pollock's Male and Female (1942). Pollock (1912–1956), an American artist born in Wyoming, earned a reputation for his classic drip paintings. He was instrumental in creating a new concept of art in which exuberant energy and motion were made visible (*39*). Pollock created his paintings on the floor rather on an easel, thereby enabling him to use his entire body to pour paint on the canvas. Pollock believed that artists did not need to go outside themselves for subject matter. He advocated that artists tap the unconscious mind, an art perspective that came to be known as abstract expressionism (*40*).

Male and Female engages the viewer quickly because of its vibrant colors and emotional brushwork (*2*). The painting is characterized by skeins of dripping paint and by scratching and scraping that expose the canvas. The painting consists of 2 centrally placed, youthful figures. While the eyelashes and curvaceous forms of the figure on the left and the more angular form and numbers on the figure on the right predispose one to assume that the former is female and the latter is male, the sex of each figure remains ambiguous. The figures can be seen as facing each other, both facing to the left, or both turning their backs to each other. The figures stand in the midst of a complex network of signs, numbers, and splattered paint.

Was Pollock possibly portraying the complex communication patterns between the sexes? Communication, defined as exchange of information, is key to interactions and sexual health. However, despite being crucial for preventing HIV infection and other STDs, communication about sexual health has always been emotionally charged (*41*). Different factors affect women's communication about sexual health or the use of condoms with their male

partners. Most commonly reported are guilt and shame, fear of personal violence, abandonment, economic repercussions, and harsh judgment (*42*,*43*). Cultural expectations to be passive make it more difficult for women to take responsibility for their sexual health and prepare for possible sexual encounters (*44*). A broader spectrum of behavioral skills and biomedical interventions, such as microbicides, offers alternatives to avoidance of risk-producing situations (*45*). Nevertheless, sex partners still need to talk about safe sex, encourage mutual testing for HIV and other STDs, and discuss test outcomes and preventive and treatment regimens.

Did Pollock intend Male and Female to teach about sexual health communication? HIV risk-reduction interventions teach communication skills as an important component in prevention and control of HIV and other STDs (*46*). These interventions seek to change behavior by providing information about risk reduction, partner communication, sexual assertiveness, sexual negotiation, refusal to have unsafe sex, and avoiding or minimizing partner abuse and violence during disclosure of STDs (*47*).

Communication about sexual health is also important between parents and children. Many parents find it difficult to talk with their children about safe sex. One father jokingly remarked that he believed in making the world safe for his children, but not for his children's children because he did not think his children should have sex or talk about sex. Educational interventions can help parents feel more comfortable talking with their children about sexual health (*48*). Some believe that communication about sexual health can have adverse behavioral outcomes, such as increased sexual activity, risky sexual behavior, or earlier sexual debut. However, HIV interventions to change risk behavior have not been associated with unintended negative consequences (*49*).

Duchamp hid the nude woman behind closed doors, and possibly, Pollock portrayed the communication dynamics between the sexes, expressing social expectations about sexual behavior and health. Because sex and sexuality are sensitive subjects, policymakers hesitate to discuss them. But leadership in policy and science is needed to prevent and control transmission of infections. The spread of HIV has necessitated discussion of sexual behavior and health to promote preventive behavior and connect people with appropriate care. Breaking the silence and stigma that surround sexual behavior communication enhances sexual health (*50*). Without addressing societal barriers, prevention and treatment interventions cannot achieve their full potential.

Scientists and artists examine and portray the pleasure and pain of sexual intimacy and sexual health. Artists portray human sexuality on canvas, in sculpture, and through art installations. They use various techniques, portray different degrees of sensuality, and evoke multiple emotions. When artists deal with human sexuality, we can learn about sexual health. This bridging process is needed because discussing sexual health is challenging, given the private nature of sexual behavior, the social stigma associated with many sexual practices and with HIV/AIDS and STDs, and the moral values associated with sexual behavior. Fighting silence and stigma and promoting empowered relationships can control infection. Addressing social factors that facilitate transmission of STDs and HIV/AIDS and advocating for strong leadership are necessary.

Medical illustrations, as used in textbooks, depict clinical manifestations of disease to teach about prevention and treatment. However, fine art can provide useful starting points for teaching and generating discussion. Art and science remind us of the joy and pain of human intimacy, the need for responsible sexual behavior, and the importance of prevention and control of HIV/AIDS and other STDs.

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Information about the authors and additional resources are available at http://www.cdc.gov/ncidod/EID/vol12no11/06-0804.htm#app

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Panton-Valentine Leukocidin–producing *Staphylococcus aureus*

To the Editor: Panton-Valentine leukocidin (PVL) is a cytotoxin produced by *Staphylococcus aureus* that causes leukocyte destruction and tissue necrosis (*1*). Although produced by <5% of *S. aureus* strains, the toxin is detected in large percentages of isolates that cause necrotic skin lesions and severe necrotizing pneumonia (*2*). Although commonly associated with community-acquired methicillin-resistant *S. aureus* (CA-MRSA) (*3*), several outbreaks due to methicillin-susceptible *S. aureus* (MSSA) have also been reported (*4*–*6*). We describe an outbreak of cutaneous infections caused by PVL-producing MSSA that affected 6 of 11 members of 2 related families.

During a period of 6 months, a cluster of *S. aureus* skin and soft tissue infections occurred in 2 families in Jerusalem, Israel, that were related through the mothers, who are sisters. The event started with the 4-year-old boy of family A, who had 5 episodes of skin infections, including 2 episodes of perianal abscesses that required drainage and hospitalization. Culture of pus grew MSSA that was resistant to erythromycin and clindamycin. Subsequently, recurrent abscesses and cellulitis developed in the boy's father's legs, and his mother had severe periorbital cellulitis that required hospitalization and surgical drainage. Approximately 1 month later, a 9-year-old boy in family B had severe cellulitis and abscess around his knee that required hospitalization and surgical drainage. Subsequently, infections developed in 2 more children in family B: 1 had a finger pulp-space infection and the other cellulitis of the lower abdomen. All pus cultures grew *S. aureus* with identical susceptibility patterns. The cases are summarized in the Table.

Following these events, the families consulted the infectious diseases clinic at the Hadassah-Hebrew University Medical Center in Jerusalem. Since the clinical isolates were not available, nasal cultures were obtained from all family members. *S. aureus* was isolated from all the affected members of family A and from the parents and the 2 boys in family B. All 7 isolates were subjected to pulsed-field gel electrophoresis (PFGE) after digestion with *Sma*I. All except 1 had identical band patterns and the same antimicrobial drug susceptibilities as the clinical isolates. The presence of PVL genes was examined by PCR as previously described (*2*) and was detected only in the isolates with identical PFGE patterns. The families were advised to apply mupirocin nasal ointment twice a day for 5 days and to bathe with 4% chlorhexidine scrub for 1 week (*7*). At 7 months of follow-up, no new cases of skin infection had occurred in either family. An epidemiologic investigation was undertaken by the local department of health to determine if 3 kindergartens and 2 schools attended by the 7 children had an increased incidence of staphylococcal skin disease. No evidence of unusual disease was found.

We describe here the first confirmed cases of PVL-producing *S. aureus* infections in Israel. Maier et al (*8*) recently described 2 cases of similar infections that occurred in German tourists after visiting the Dead Sea area, but since these infections were caused by MRSA, it is probable that the isolates were genetically distinct from the strain described here. In addition, to the best of our knowledge this is the first description of transmission of PVL-producing MSSA between related families. Previous reports described community-related outbreaks that occurred within families (*6*,*8*,*9*), between schoolmates (*4*), and between football team players (*10*). The exact route of transmission was not identified in some of these cases but it was presumed to have been close contact leading to skin (*10*) or nasal (*4*) colonization and subsequent active infection. In our report, the PVL-producing *S. aureus* clone was detected in

Table. Clinical and microbiologic data of the outbreak*

No.	Family	Sex/ age(y)	Clinical manifestations	Pus cultures	Nasal cultures	PFGE pattern/PVL
1	A	F/33	Periorbital cellulitis	MSSA, Ery/clin- R	MSSA, Ery/clin- R	I/P
2	A	M/36	Leg cellulitis/abscess	None	MSSA, Ery/clin- R	I/P
3	A	M/4	Perianal abscess	MSSA, Ery/clin- R	MSSA, Ery/clin- R	I/P
4	A	F/2	None		N	
5	A	F/1	None		N	
6	B	F/37	None		MSSA, Ery/clin-R	I/P
7	B	M/38	None		MSSA, Ery/clin-R	I/P
8	B	M/9	Knee cellulitis/abscess	MSSA, Ery/clin- R	MSSA, Ery/clin- R	I/P
9	B	M/4	Finger-pulp infection	None	MSSA, Ery/clin- S	D/N
10	B	F/3	Lower abdomen cellulitis	None	N	
11	B	F/3	None		N	

*PFGE, pulsed-field gel electrophoresis; PVL, Panton-Valentine leukocidin; F, female; MSSA, methicillin-sensitive *Staphylococcus aureus*; Ery, erythromycin; clin, clindamycin; R, resistant; I, identical strain; P, positive; M, male; N, negative; S, sensitive; D, different strain.

nasal cultures in 6 of the 11 members of the 2 families. In this niche, it was able to persist and cause a series of infections in a relatively large number of family members. Even though the *S. aureus* isolated from active lesions were not available for testing, the recovery of identical PVL-positive organisms from nasal cultures strongly suggests the presence of a pathogenic clone that probably caused the recurrent infections in the 6 affected family members. Our investigation highlights the high transmissibility of this PVL-producing *S. aureus* clone, its high attack rate, and its virulence. The intervention in this outbreak might have prevented not only subsequent recurrences of cutaneous infections but also further spread of this clone and the manifestation of even more serious infections such as necrotizing pneumonia. Increasing awareness among community-based healthcare providers of PVL-producing *S. aureus* infections is important to facilitate rapid and adequate response in similar clinical events in the future.

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Chikungunya Fever, Hong Kong

To the Editor: Chikungunya virus disease, caused by a mosquitoborne alphavirus, is endemic to Africa and Southeast Asia. It typically causes an acute febrile illness, with joint pain and a skin rash. Chronic arthropathy may develop (*1*,*2*). No treatment or vaccine is available, and relatively little research has been conducted into its pathogenesis, compared with that of other arboviruses, such as dengue. Recent reports have described a massive outbreak of chikungunya disease occurring on islands in the Indian Ocean, off the east coast of Africa (*1*). Reemergence of chikungunya has also been reported from Indonesia (*2*).

During March 2006, a 66-year-old Chinese man from Hong Kong visited Mauritius where he was bitten by mosquitoes 2 days before returning to Hong Kong. On the return trip, he experienced fever (39°C), severe finger joint and muscle pains, mild headache, and a skin rash, and he sought treatment at the Prince of Wales Hospital (PWH) Infectious Diseases Clinic on the second day of his illness. Physical examination showed a generalized erythematous rash over the trunk and limbs and petechiae over the lower limbs. Mild finger joint stiffness was observed, but no joint swelling. No lymphadenopathy or eschar was detected. Level of C-reactive protein was elevated at 10.4 mg/L. Results of screens for malaria and dengue were negative. Results of other routine assessments were unremarkable. His symptoms subsided gradually within a week.

Serum specimens taken on days 2 and 6 were positive for chikungunya virus RNA by in-house reverse transcription (RT)-PCR at the Public Health Laboratory Service (PHLS) (targeting the nonstructural protein-1 [NSP-1] gene) and PWH laboratory (targeting both NSP-1 and the envelope glycoprotein [E1] gene). An

additional serum sample taken on day 8 of illness, received by PHLS only, was also positive for chikungunya RNA. Both laboratories confirmed RT-PCR results by sequencing. At PWH, phylogenetic analysis was performed to determine the likely origin of the virus. In-house immunofluorescent slide serologic assays developed at PHLS found chikungunya immunoglobulin G (IgG) titers <10, 160, and 320 in the serum samples taken on days 2, 6, and 8 of illness, respectively, and detected chikungunya IgM in the day 8 serum. The acute cytokine immunologic response to this virus was also tested (online Appendix available from http://www.cdc.gov/ncidod/EID/vol12no11/06-0574_app.htm).

Sequencing and phylogenetic analysis was consistent with an imported infection, almost certainly originating from the current chikungunya outbreaks in the Indian Ocean. Phylogenetic analyses of the NSP-1 and E1 regions, indicated that this virus is most closely related to previous African rather than South-east Asian chikungunya viruses (see online Appendix Figures 1 and 2, available from http://www.cdc.gov/ncidod/EID/vol12no11/06-0574_appG1.htm and http://www.cdc.gov/ncidod/EID/vol12no11/06-0574_appG2.htm). The persistence of viremia up to at least day 8 of illness was unusual. Standard texts state that viremia may be present during the first 2–4 days of illness, with neutralizing antibodies appearing by days 5–7 (*3*).

The most striking finding from the cytokine analysis (Table) is the high level of interferon-γ (IFN-γ)– inducible protein-10 (IP-10/CXCL-10), up to 26 and 16 times the upper limit of the normal range at days 2 and 6 after disease onset, respectively. Serum concentrations of interleukin-8 (IL-8), monocyte chemoattractant protein (MCP) 1 (MCP-1) and monokine induced by IFN-γ (MIG/ CXCL9) are also elevated in both samples. Notably, serum IFN-γ, tumor necrosis factor-α (TNF-α), and IL-1β, 6, 10, and 12 concentrations remain within normal limits in both samples, although the concentrations at local inflammatory sites (e.g., joints) are unknown. CXCL10 and MCP-1/CCL2 concentrations decreased during clinical recovery. Thus, the cytokine profile demonstrates that the levels of Th1 chemokine CXCL10 was highly elevated and that the levels of chemokines IL-8/CXCL8, CCL2, and CXCL9 were moderately elevated. In contrast, IFN-γ and other inflammatory/Th2 cytokines were not elevated during the illness.

Interpretation of the significance of these cytokine results is necessarily speculative. Some comparison can be made with other viral infections. In severe acute respiratory syndrome–associated coronavirus (SARS-CoV) (*4*,*5*) and H5N1 influenza (*6*) infections, very high blood levels of CXCL10 and moderately high CCL2, CXCL9, and CXCL8 concentrations, or their enhanced expressions in vitro, have been reported. In dengue fever, which has similar clinical manifestations as chikungunya fever, only elevated CXCL8, IL-6, IL-10, and TNF-γ concentrations have been shown consistently (*7*,*8*), although CXCL10 expression has not been studied.

The function of CXCL10 is to act as a chemoattractant for Th1 cells in the activation of cell-mediated immune response. Its expression can be up-regulated by the Th1 cytokine IFN-γ during acute inflammation. CXCL10 has been implicated in the pathogenesis of SARS-CoV and H5N1 influenza infections, in which persistently high CXCL10 concentrations seem to correlate with disease severity and progression (*4*–*6*). CCL2, CXCL9, and CXCL8, have also been found to have a pathogenic role in H5N1 influenza, SARS-CoV, and dengue infections. Notably, the level of antiviral cytokine IFN-γ was not elevated in our chikungunya case, though admittedly, this is only 1 case. This finding may represent a way that the chikungunya virus evades host defenses and may provide a rationale for the use of IFN as a therapeutic option (*9*). Such IFN therapy has been suggested and tried, experimentally, for SARS-CoV (*5*) and dengue infections (*10*).

Table. Serum cytokine profiles of patient with chikungunya infection, days 2 and 6 of illness*

	Time after onset of illness	
Serum cytokine profile (ng/L)	2 d	6 d
IFN-γ, N<15.6	UD	UD
IL-1β, N<7.2	2.4	1.5
IL-6, N<3.1	UD	1.3
IL-10, N<7.8	1.7	1.7
IL-12 p70, N< 7.8	3.6	1.4
TNF-α, N< 10.0	UD	UD
CXCL8, N<10.0	45.3	54.3
CXCL10†, N = 232–1,019	26,319	16,156
CCL2†, N = 18–152	445	257
CXCL9†, N = 37–463	1,138	1,605
CCL5, N = 10,349–46,704	24,745	60,671

*N, normal range; IFN, interferon; IL, interleukin; UD, undetected; TNF, tumor necrosis factor.
†Both 2 d and 6 d and led samples above normal range.

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Screening Laboratory Requests

To the Editor: In August 1999, the Laboratory Response Network (LRN) was established to better integrate and improve laboratory capacity for responding to public health threats (*1*). However, while experts have focused on clinical indications for testing for agents of bioterrorism, laboratory methods for microbial identification, and needs for integrated communication networks (*2–4*), little attention has been given to how sentinel laboratories can effectively screen clinicians' requests for testing pathogens designated as global health threats.

In times of crisis, clinicians often pressure laboratorians to perform testing for patients whose probability for disease is very low or for nonvalidated sample types. In 2001, a few cases of anthrax triggered large numbers of nationwide requests to test nasal swabs for *Bacillus anthracis* despite the absence of data to support this clinical practice outside epidemiologic investigations (*5*). Similarly, a false-positive result for severe acute respiratory syndrome (SARS) in 2003 from the National Microbiology Laboratory in Canada created public alarm that SARS was reemerging, when the virus was actually that of a common respiratory illness in a nursing home (*6*). The problem is further complicated when laboratories other than the LRN lack standardization, have greater access to nucleic acid amplification-based testing, and develop tests for global health threats outside a quality-regulated system. False-positive results caused by contamination or cross-reactivity with a microorganism of low virulence can disrupt a public health system, adversely affect patient care, and increase costs (*6–8*); false-negative results may prompt clinicians to discontinue containment procedures and potentially risk transmitting a virulent microorganism. At our sentinel laboratory, we recognized these challenges and took steps to promote judicious use of testing for agents designated as global health threats. We report use of an algorithm to evaluate test requests for SARS-associated coronavirus and highly pathogenic avian influenza H5N1; however, the algorithm can be used to screen testing requests for any pathogen that has potential to threaten public health.

During outbreaks of SARS and H5N1, a laboratory protocol was established to notify the on-call laboratory professional when a sample was received for testing for 1 of these pathogens (Figure). The protocol required the laboratorian to communicate directly with the clinician, using a script with questions based on criteria established by the Centers for Disease Control and Prevention, to determine the medical necessity for testing (*9,10*). Samples from patients not meeting these criteria were rejected. Testing for SARS used an in-house real-time PCR assay with a standard laboratory protocol. Samples accepted for H5N1 testing were screened by a nonspecific hemagglutinin influenza PCR assay and, if results were positive, were to be forwarded to an LRN laboratory. Positive results were to be reported only after confirmation by an LRN laboratory. Laboratory professionals were specifically trained about the sensitivity, specificity, positive predictive value, and negative predictive value of test methods in relation to sample type, time between symptom onset and specimen collection, and disease prevalence.

Of 41 samples (40 SARS and 1 H5N1) received for testing, 26 (63%) samples were not tested because clinician responses failed to satisfy the screening criteria. The remaining 15 (37%) samples met criteria for testing and all had negative results. In the

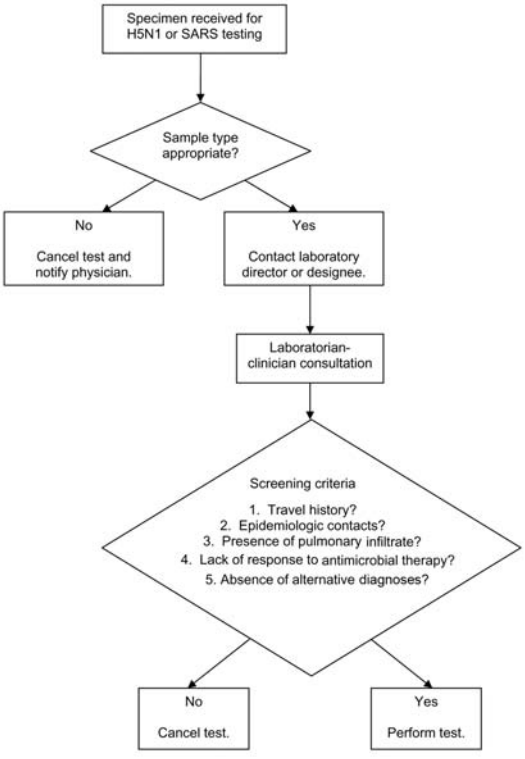

Figure. Laboratory algorithm used to screen test requests for avian influenza H5N1 or severe acute respiratory syndrome (SARS)

absence of positive results, no confirmatory testing was indicated.

Although SARS no longer poses a credible threat and human-to-human transmission of H5N1 has not been well delineated, our experiences with these 2 pathogens demonstrate how a sentinel laboratory can effectively intervene in the initial phases of a public health threat. We found that having a laboratory professional contact the clinician and systematically ask the scripted questions was a pragmatic tool for the first phase of response and resulted in cancellation of most tests. We acknowledge that optimal validation of this algorithm would require randomly selecting and testing rejected specimens during a phase of high disease prevalence. Although low disease prevalence during our study period precluded validation testing, we recommend that such testing be performed.

Our systematic approach to screening requests to test for agents with the potential to threaten global health can prevent arbitrary decision making, reduce inappropriate testing, and increase the value of laboratory consultation. The principles guiding our testing protocols for SARS and avian influenza can be generalized to future global health threats. Responsible and judicious use of diagnostic testing will be crucial for minimizing the risk of providing clinicians with misleading results that could severely disrupt the public health system and lead to an unnecessary expenditure of limited resources. With the emergence of highly pathogenic avian influenza, we anticipate further demands on laboratory and public health resources that will necessitate effective, pragmatic tools to enhance the value of laboratorian-clinician consultation before tests are performed on site or referred to an LRN laboratory.

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Malaria Outbreak in Troops Returning from French Guiana

To the Editor: In January 2005, the chief surgeon in a squadron of French policemen reported a cluster of *Plasmodium vivax* malaria attacks in troops returning from a 108-day operation in French Guiana. We conducted a retrospective cohort study to describe the malaria attacks and determine factors related to them.

A self-administered questionnaire was drawn up, with questions concerning operations in French Guiana (dates, locations) and preventive measures implemented against malaria. A malaria case was defined by the association of clinical signs and *Plasmodium* parasites in blood smears or quantitative buffy coat tests (per definition of military epidemiologic surveillance).

The 40-person mission in French Guiana (Operation Anaconda) took place from July 26, 2004, to November 6, 2004 (108 days of exposure). This mission against clandestine gold panning was conducted in a deep-forest environment where the troops were temporarily housed in villages of Brazilian gold panners. Occasionally, they washed themselves late in the evening in stagnant water near the river and patrolled outside during maximum biting periods. All troops received a chemoprophylaxis (doxycycline 100 mg daily) during the mission and for 4 weeks afterward.

From July 2004 through January 2005, 10 persons had ≥1 malaria attacks (attack rate 25%) for a total of 18 malaria attacks (incidence 13/100 person-months of exposure). *P. vivax* was isolated for 17 attacks and *P. falciparum* for 1 attack (Figure). Five patients had 1 malaria attack, and 4 patients had up to 3 relapses. Six patients had a malaria attack while receiving doxycycline.

Regarding chemoprophylaxis compliance, 34% reported missing <1 dose per week and 32% were fully compliant. The troops did not have permethrin-impregnated battlefield uniforms as do soldiers in the French Army. They had to impregnate their own uniforms with permethrin. Only 37% said they always wore clothing that fully covered them during the mission, and 86% reported having frequently used a repellent. All reported having slept under mosquito nets. No association was found between malaria attacks and regular chemoprophylaxis intake or use of repellents. Only 1 operation in French Guiana was associated with the risk of experiencing malaria attacks: 39% of troops located in Sikini had at least 1 malaria attack versus 7% of troops in other areas (relative risk: 5.9 [95% confidence interval 0.8–41.7]).

The incidence rate for this study was 10 times higher than the maximum incidence rate observed for French troops deployed in Côte d'Ivoire (1.3/100 troop-months in 2004). During an earlier Operation Anaconda, 37 of 62 persons deployed near the Sikini area had ≥1 malaria attacks (attack rate 61%). Of these, 30 had ≥1 attacks caused by *P. vivax*; occasionally an attack was associated with *P. falciparum* (*1*).

Our results suggest that the Sikini area was the high-risk area for malaria transmission (although the large confidence interval reflects a lack of power in our analysis). The operation dates (15–28 September) are compatible with the duration of the first cases of malaria occurrence.

French Guiana is the only French territory, except for Mayotte, where malaria is endemic, with nearly 5,000 cases per year, occurring mainly along the rivers bordering Suriname and Brazil (*2*). The highest frequencies of malaria appear during the dry season (September to December) in French Guiana (*3*), but no seasonality was described near the Brazilian border (*4*).

The Sikini area is located near the Oyapock River (Brazilian border). The mean annual incidence in Amerindians there is 48.6%, mainly due to *P. falciparum* (incidence 24.8%) and *P. vivax* (incidence 25.9%) (*2*).

P. vivax malaria incidence has increased in the Oyapock region, from 30% in 1987 to 50% in 2000–2004 (*2*,*4*–*7*). French troops were deployed in an area where parasite circulation was high. Troops had

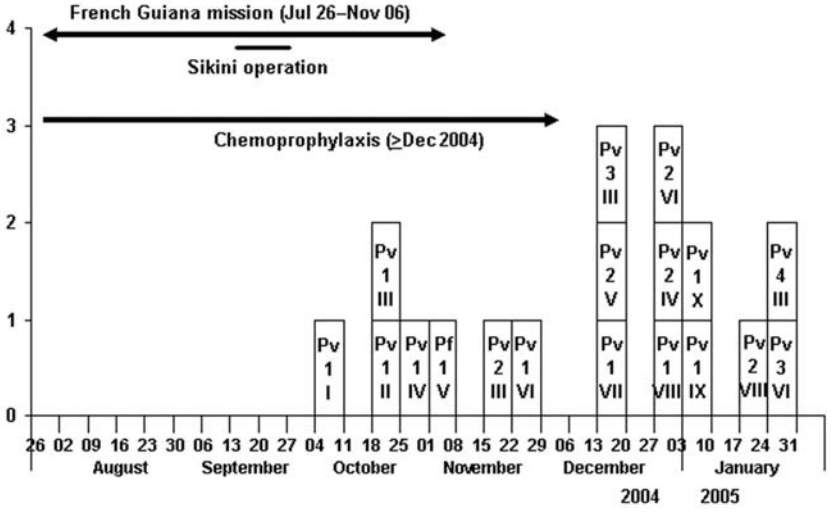

Figure. Epidemic curve of malaria attacks. Pv, *Plasmodium vivax*; Pf, *P. falciparum*; 1, access no.; I, case no.

contacts with clandestine gold panners, mainly Brazilian illegal residents. This population, in which malaria incidence is almost impossible to evaluate, comes from Amapa State, where the incidence of malaria is increasing (*5*). In 2003, 60.9% of patients with malaria cases at Cayenne Hospital had a Brazilian name compared with 35.4% in 2000 (*6*). Also, the gold panners diverted the river and built basins where vectors could easily multiply (*7*).

Initial malaria attacks were treated with chloroquine or quinine. Five patients experienced ≥1 relapses (maximum 3 relapses). The relapses were treated with 50-mg daily doses of primaquine for 4 patients and by chloroquine for the fifth patient. Two patients had relapses after receiving primaquine. Primaquine resistance information was not available. However, resistance to primaquine has emerged in *P. vivax* strains (*8*).

We recommended that pre-impregnated battlefield uniforms be available for French policemen and chemoprophylaxis adherence be reinforced by directly observed intake by supervisory staff. Relapses of *P. vivax* malaria are a major therapeutic problem, particularly after primaquine therapy.

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Plasmodium vivax Malaria Relapses after Primaquine Prophylaxis

To the Editor: Standard treatment of patients with *Plasmodium vivax* malaria includes chloroquine, followed by primaquine terminal prophylaxis. Reports of true primaquine failure and subsequent *P. vivax* relapse are unusual; most suspected cases can be ascribed to poor patient adherence, recrudescence of a chloroquine-resistant strain, or *P. vivax* reinfection. We report a case of *P. vivax* malaria relapse after therapy with quinine, doxycycline, and primaquine, and again after treatment with chloroquine and primaquine. *P. vivax* relapses after primaquine treatment are exceedingly rare in travelers to South America and are a serious therapeutic challenge. Our patient was subsequently treated with weekly, single-dose chloroquine without recurrence of symptoms.

A 77-year-old man had fever and chills 2 weeks after returning from Brazil. These symptoms were accompanied by sweating, fatigue, and a mild, productive cough. Review of systems was notable for dark, concentrated urine and a 10-lb weight loss. The patient's 25-day journey included Salvador, Manaus, and a 2-day stay in the Amazon River basin. He did not take malaria prophylaxis during his trip.

On physical examination, the patient was afebrile with blood pressure of 90/53 mm Hg. Cardiovascular, pulmonary, and abdominal examination results were unremarkable. Several petechiae were noted on both lower extremities. Laboratory tests showed the following: leukocyte count 6,300 cells/μL, hemoglobin level 13.7 g/dL, platelet count 40,000 cells/μL, serum creatinine level 1.2 mg/dL, serum alanine aminotransferase level 63 IU/L, and serum

aspartate aminotransferase level 56 IU/L. Thick and thin peripheral blood smears revealed *P. vivax* with a parasitemia level of 0.67%. Although the existence of chloroquine-resistant *P. vivax* in Brazil is debatable, the patient was conservatively treated with quinine, 650 mg, 3×/day and doxycycline, 100 mg, 2×/day for 7 days, followed by primaquine terminal prophylaxis, 30 mg/day for 30 days with complete resolution of symptoms.

In the absence of travel abroad, the patient experienced similar symptoms 5 months later. On the basis of thick and thin peripheral blood smear examination, a relapse of *P. vivax* malaria was diagnosed. He was given chloroquine, 2.5 g over 3 days, followed by primaquine, 30 mg/day for 30 days. Again, the patient's symptoms resolved.

Four months after treatment (9 months after the initial episode), the patient experienced the abrupt onset of fever, chills, and dark urine. He had a leukocyte count of 5,900 cells/μL, a hemoglobin level of 14.0 g/dL, and a platelet count of 117,000 cells/μL. Repeat thick and thin blood smears showed *P. vivax* with a parasitemia level of 0.993%. Therapy with chloroquine was initiated (2.5 g over 3 days), and symptoms resolved. Repeat blood smears 4 days later were negative for *P. vivax*. In lieu of yet another course of terminal prophylaxis with primaquine, the patient was given chloroquine, 300 mg/week for 4 months; he has been asymptomatic for an additional 2 months.

Even before Food and Drug Administration approval of primaquine in 1951, primaquine failure was documented in experimental cases of the Chesson (tropical) *P. vivax* strain (*1*). Additional reports soon followed, citing dosing differences as the likely reason for *P. vivax* relapse. Baird and Hoffman summarized cases of primaquine failure over nearly 3 decades, noting that 26 (25%) of 103 patients given primaquine, 15 mg/day for 14 days, relapsed, while infection returned in only 1 (3.9%) of 26 patients given 22.5–30 mg/day (*2*). Among 50 patients treated for *P. vivax* malaria in Brazil, total primaquine dose per patient was the only variable in relapse; 7 relapses occurred in patients who received 2.76 mg/kg, while those who received 3.35 mg/kg remained free of infection (*3*). As a consequence, patients weighing >70–80 kg should receive 0.5 mg/kg/day (*2*).

The issue of primaquine resistance in *P. vivax* remains unresolved for several reasons. First, the organism cannot be propagated in vitro, and injection of *P. vivax* into nonhuman primates is required for analysis (*4*). Second, the pharmacokinetics of primaquine are poorly understood. Despite standard dose administration, 1 study suggested substantial interethnic differences in peak plasma concentrations of primaquine and its major metabolite, carboxyprimaquine (*5*). Finally, confounding factors such as drug dosing and patient compliance have complicated most failure reports.

Our patient initially received quinine and doxycycline, which excluded a chloroquine-resistant infection. In addition, he completed a primaquine regimen of 10.8 mg/kg, which is twice the current recommended dose. In the absence of reexposure, the patient had a relapse 5 months later. His condition was treated with chloroquine and again with high-dose primaquine. He reported strict adherence to the treatment regimen, citing the fastidious use of a weekly pill box as evidence. Despite these measures, another relapse occurred 4 months later. This patient's course suggests *P. vivax* primaquine failure and possible resistance. When high-dose regimens of primaquine (total 5–6 mg/kg) fail, suppressive doses of chloroquine, 300 mg/week for several months to years may be considered. Our patient received chloroquine therapy, 300 mg/week for the past 4 months without evidence of recurrence.

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Avian Influenza and US TV News

To the Editor: Scholars have routinely noted ways in which scientific inquiry is isolated from public life and popular attention and have bemoaned relatively low levels of scientific literacy among lay audiences (*1*–*3*). While public understanding of science in the United States and elsewhere undoubtedly is not at the level desired by most scientists, apparent interest and hunger to learn are high for certain issues. These issues represent public communication opportunities.

Avian influenza is now such an issue. Although the risk for pandemic human influenza stemming from the avian influenza H5N1 virus is thought to be relatively low (*4*), media coverage of the disease, at least superficial and episodic coverage of disease incidence, has been dramatic. Aside from existing coverage, however, what type of coverage should the issue receive according to viewers? Are they interested in the issue, if at all, as a matter of scientific inquiry or simply as a sensational threat to individual survival?

We report here relevant results from a national survey of local television news viewers in the United States. Evidence from an Internet-based survey conducted in May 2006 suggests that viewers not only think that the potential direct impact of avian influenza on their own lives should be covered by reporters but also have interest in scientific investigation of the disease.

Working with Survey Sampling International (available from www.surveysampling.com), we recruited by email a nationally representative sample of regular television news viewers. Potential respondents were offered the chance to win a cash prize. Only those ≥18 years of age and those who watched local television news show at least twice a week in recent months contributed to the final survey. We report here data from the 2,552 respondents who met those criteria and who answered all relevant questions.

Participants represented a reasonable cross-section of the general US population of television news viewers. Participants were 18–90 years of age (mean age 52, SD = 15.45). Educational attainment was mixed: 37% reported having completed at least a 4-year undergraduate degree, and 63% had completed <4-year degree. The final sample was 87% Caucasian, 8% African American, and 2% Asian; 8% also identified themselves as Hispanic or Latino. Approximately 54% of the sample was female, and 11% reported that they work for an organization directly involved in science.

When offered a 7-point scale that ranged from "not at all important" to "very important" to describe the priority that local television news should assign to addressing the "direct impact" of avian flu on one's own life and the lives of others, ≈80 % chose ≥5. Approximately 42% of respondents chose the highest level, indicating it was very important for local television news to cover this angle of the story. Regarding deeper perspectives on the story, ≈81% of respondents chose ≥5 on the 7-point scale of importance when asked about potential coverage of how avian flu spreads and why scientists are finding it difficult to contain; 41% of respondents thought that it was "very important" that television reporters explicitly discuss that aspect of the issue. Moreover, 69% of respondents, by offering ≥5 on the 7-point scale, thought the television news should focus on the connection of avian flu to other issues, such as business and travel. Clearly, we are living in a time in which news audiences would tolerate much more than the soundbites and superficial coverage often offered with regard to infectious disease research.

Equally as striking are the demographic characteristics of those who believe that local television news should cover the process of scientific discovery in this arena. We conducted a simple regression analysis to predict 1 of the items noted above, i.e., perceived importance of television news discussion of how avian flu spreads and of the efforts of scientists. We used formal employment with a scientific institution, level of educational attainment (a 5-level variable treated here as interval), and reported conversation with others about science in recent months as predictors. Educational attainment actually bore a negative relationship to interest in such coverage, $\beta = -0.14$, $p<0.01$, and formal affiliation with a scientific institution bore no statistically significant relationship, $p>0.10$. (Past conversation about science bore a positive relationship, $\beta = 0.06$, $p<0.01$.)

Results suggested a prime opportunity for public communication efforts not just because of issue timeliness but also because of apparent widespread hunger for information among the US television news viewers. Health and science communication professionals could address this interest and desire to boost popular awareness of epidemiologic and medical inquiry.

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Resistance to Dihydroartemisinin

To the Editor: The emergence of widespread resistance to chloroquine and sulfadoxine-pyrimethamine in Africa has caused a sharp rise in deaths from malaria. The World Health Organization therefore urgently recommends replacement of these drugs, particularly with combinations that include an artemisinin compound (AC) (*1*). In 2006, although >40 countries have adopted artemisinin-based combination therapies as their first-line treatment for malaria, only a few of these countries actually use these combination therapies because of limiting factors such as high cost (*2*). When used as monotherapy, ACs are associated with high rates of recrudescence, possibly because of their short elimination half-lives (*3*). Most artemisinin-based combination therapies contain, in addition to ACs, a partner drug against which resistance has already developed (e.g., mefloquine, amodiaquine, lumefantrine); reports of relatively low efficacy of the combination artesunate-amodiaquine have been recently published (*4*). In 2005, Jambou et al. claimed to have found the first cases of in vitro *Plasmodium falciparum* resistance to ACs (*5*).

We assessed the in vitro susceptibility to dihydroartemisinin (dhART), the biologically active metabolite of artemisinin derivatives, of *P. falciparum* isolates from travelers returning to France from various African countries during 2004–2006. In addition, we searched for polymorphism in the *P. falciparum* adenosine triphosphatase-6 (*PfATPase6*) gene, which was reported to be associated with in vitro artemether resistance (*5*). We also studied polymorphism (a 3-bp indel) in the gene of the ABC transporter G7, which was reported in 2005 to be associated with in vitro response to artesunate (*6*).

Determination of in vitro dhART susceptibility by using the isotopic semimicrotest method (*7*) was successful for 397 isolates. The most represented countries were Cameroon (17%), Côte d'Ivoire (14.5%), Mali (12%), Comoros Islands (8.5%), and Senegal (6.5%). Patients were ≤75 years of age (mean 31, SD 17 years), and the male:female ratio was 1.5:1. The 50% inhibitory concentration (IC_{50}) values ranged from 0.02 to 31.8 nmol/L, with a geometric mean of 1.31 nmol/L and a median of 0.68 nmol/L. IC_{50} values were <1 nmol/L for 264 isolates, 1–10 nmol/L for 127, and >10 nmol/L for 6. Thus, some isolates showed a diminished susceptibility to dhART, but only 1 isolate had an IC_{50} >30 nmol/L (31.8 nmol/L).

DNA sequencing of 900-bp and 240-bp PCR products, including the 769 and the 243/263 *PfATPase6* codons, respectively, was performed in a subsample of 154 isolates. All isolates had the S769 wild codon except 1 susceptible isolate (IC_{50} = 0.83 nmol/L), which had a S769N mutant type codon (Table). We found no polymorphism in codon 263. This position may be scrutinized to monitor anticipated artemisinin resistance, according to a recently published structure-function study (*8*). Conversely, we found 2 isolates that had IC_{50} values of 4.2 nmol/L and

Table. Polymorphism in *PfATPase6* and G7 genes and in vitro susceptibility to dihydroartemisinin of 154 *Plasmodium falciparum* isolates*

Gene	Predicted products	Position	Amino acid	Nucleotide change	No. isolates	Dihydroartemisinin IC_{50} (nmol/L)
ATPase6	Sarcoplasmic reticulum calcium-transporting ATPases	769	S	AGT	153	0.1–31.8
			S→N	AAT	1	0.83
		263	L	TTA	154	0.1–31.8
			L→S	TCA	0	
		243	H	CAT	152	0.1–31.8
			H→Y	TAT	2	4.2; 6.4
G7	ABC transporter	1,390	Wild	$(AAT)_4$	69	0.1–25.9
			Mutant (3-bp indel)	$(AAT)_3$	85	0.15–31.8

**PfATPase, Plasmodium falciparum* adenosine triphosphatase; IC_{50}, 50% inhibitory concentration.

6.4 nmol/L and that showed an H243Y mutant type codon. The role of such a polymorphism appears unclear. We found no association between the 3-bp indel in G7 and in vitro dhART susceptibility because mutants were regularly distributed in highly susceptible isolates and in isolates having a diminished susceptibility.

For our samples obtained during 2004–2006, the geometric mean IC_{50} value for dhART was very close to values found in Cameroon during 1997–1998 (mean dhART IC_{50} = 1.11 nmol/L) (*9*), in Senegal in 2001 (mean artemether IC_{50} = 1.3 nmol/L) (*5*), and in Republic of Congo during 2005–2006 (mean dhART IC_{50} = 1.02 nmol/L) (*10*). Ringwald et al. observed a narrower range of IC_{50}s, but their series included only 65 samples (*9*). Previous comparisons between ACs suggested that dhART is 1.7 times more potent than artemether against *P. falciparum* (*9*). Thus, the highest IC_{50} value for artemether observed by Jambou et al. in Senegal (44.7 nmol/L) (*5*) is comparable to the highest IC_{50} value for dhART in our series (31.8 nmol/L). The resistance levels of ACs are still undefined. For artemether, Jambou et al. used a threshold of 30 nmol/L to evaluate the association between the S769N mutation and in vitro susceptibility. The presence of *ATPase6* S769N was not associated with diminished in vitro susceptibility in our series. Conversely, the only S769N mutant that we observed was found in a fully susceptible isolate. Thus, we confirmed that polymorphism exists in this gene in positions 769 and 243, but we did not prove an association between these point mutations and resistance to ACs. Similarly, our results did not support the hypothesis of an association between the 3-bp indel in G7 and resistance to ACs.

ACs, considered the most important class of antimalarial drugs, merit close surveillance for susceptibility. Continued monitoring of the efficacy of their associated partner drugs also appears to be essential.

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Real-time PCR for *Francisella tularensis* Types A and B

To the Editor: *Francisella tularensis*, the etiologic agent of tularemia, is highly infectious and considered a potential bioweapon (*1–3*). Although 4 subspecies of *F. tularensis* are recognized, most cases of tularemia are due to infection by subsp. *tularensis* (type A) or *holarctica* (type B). North America is the only region where both type A and type B cause human disease. Subspecies *novicida* is also found in North America, but it is of reduced virulence. Disease incidence attributable to either type A or type B is

essentially unknown because the traditional method for classification of these subspecies is glycerol fermentation, which requires culture recovery (*4*). *F. tularensis* is fastidious and slow growing, with isolates recovered in a small percentage of cases.

We developed real-time TaqMan PCR assays for classification of *F. tularensis* type A and type B after *F. tularensis* is identified by culture or, in the absence of culture, by a PCR method such as the *F. tularensis* multitarget TaqMan assay (*5*). The type A TaqMan assay targets *pdpD*, which is present in type A, almost entirely absent from type B, and contains a 144-bp insert in *novicida* (*6*,*7*) (F: 5′-GAGACATCAATTAAAAGAAG-CAATACCTT-3′; R: 5′-CCAAGAG-TACTATTTCCGGTTGGT-3′; probe: 5′-AAAATTCTGC"T"CAGCA-GGATTTTGATTTGGTT-3′). The type B assay targets a junction between IS*Ftu2* and a flanking 3′ region (GenBank AY06) (F: 5′-CTTGTACTTTTATTTGGCTACT-GAGAAACT-3′; R: 5′- CTTGCTTG-GTTTGTAAATATAGTGGAA-3′; probe: 5′- ACCTAGTTCAACC"T"-CAAGACTTTTAGTAATGGGAAT-GTCA-3′). In type A and *novicida*, IS*Ftu2* is absent from this position (*8*). Oligonucleotides were designed with Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA). Probes were synthesized with a 5′ 6-carboxy-fluorescein reporter and an internal quencher (either BHQ1 [type A] or QSY-7 [type B]) at the nucleotide position indicated by the quotation marks.

Assays were optimized by using 1 ng of type A (strain SchuS4) or type B (strain LVS) DNA on the LightCycler 1.2 (Roche Applied Science, Indianapolis, IN, USA). Optimized concentrations (20 μL final volume) were 1× LightCycler Fast Start DNA Master Hybridization Probe mix (Roche), 750 nmol/L primers, 200 nmol/L probe, 5 mmol/L $MgCl_2$ and 0.5 U uracil-DNA glycosylase. PCR conditions were 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 10 s, and 65°C for 30 s, then 45°C for 5 min. Cycle threshold (C_t) values were calculated by using the second derivative maximum method with the y-axis at F1/F3 (LightCycler software version 3.5).

Sensitivity of each assay was assessed by using 10-fold serial dilutions (100,000 to 1 genomic equivalents [GE]) of SchuS4 or LVS DNA. Testing was performed in triplicate, with a reproducible detection limit of 10 GE for both assays. Specificity of each assay was tested with 1 ng of DNA from a panel of 62 *Francisella* isolates (online Appendix Table, available from http://www.cdc.gov/ncidod/EID/vol12no11/06-0629_appT.htm) and 22 non-*Francisella* isolates (*Acinetobacter, Bacillus, Brucella, Corynebacterium, Enterobacter, Enterococcus, Escherichia, Haemophilus, Klebsiella, Legionella, Proteus, Pseudomonas, Serratia, Staphylococcus, Streptococcus,* and *Yersinia* species). Isolates were grown, DNA purified, and quantified as previously described (*5*). Specificity was also evaluated with DNA (2 μL) extracted as previously described from *Francisella*-like tick endosymbionts of *Dermacentor variabilis* and *Francisella*-like soil bacteria (online Appendix) (*9*,*10*). The type A assay recognized all type A isolates with an average C_t value of 17.9 (n = 19). The type B assay detected all type B strains with an average C_t value of 17.1 (n = 21). Neither assay displayed cross-reactivity with *F. tularensis* subsp. *novicida* (n = 7), *F. philomiragia* (n = 15), *Francisella*-like tick endosymbionts (n = 3), *Francisella*-like soil bacteria (n = 7) (Appendix), or non-*Francisella* spp. (n = 22).

To evaluate the ability of the type A and type B TaqMan assays, in conjunction with the multitarget assay, to identify *F. tularensis* and classify subspecies in primary specimens, human, animal, and tick samples were tested (Table) available from DNA was extracted from 200 μL fluid, 25 mg liver, and 10 mg spleen or lung by using the QIAamp DNA MiniKit (Qiagen, Valencia, CA, USA) and 1 μL tested. Multitarget PCR conditions were as described (*5*).

The multitarget and subspecies-specific PCR assays accurately identified and classified *F. tularensis* in all specimens positive by standard diagnostic methods (Table). In addition, the type A and type B assays provided subspecies information for positive specimens in which an isolate was not recovered for glycerol fermentation testing (Table). All specimens negative by standard diagnostic methods tested negative by PCR. These preliminary results suggest that a *F. tularensis* PCR identification method, in combination with the type A and type B assays, provides the capability to identify *F. tularensis* and determine subspecies in the absence of culture.

We describe real-time PCR assays capable of classifying *F. tularensis* type A and type B and distinguishing these subspecies from the less virulent subsp. *novicida*. These assays are designed for use after *F. tularensis* has been identified by culture or by PCR. Supplemental use of these assays will allow laboratories to actively subtype *F. tularensis* isolates and primary specimens, thus providing subspecies information for a higher percentage of tularemia cases. Improved subspecies information will further understanding of the disease incidence and geographic distribution of *F. tularensis* type A and type B in North America.

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Table. Comparison of standard diagnostic methods with the multitarget *Francisella tularensis* TaqMan assay and type A and type B assays using primary specimens

Specimen	Source	*F. tularensis* identified*	Subspecies identification†	Multitarget *F. tularensis* TaqMan assay‡			Type A assay‡ (C_t value)§	Type B assay‡ (C_t value)
				IS*Ftu2*	*iglC*	*tul4*		
Lymph node aspirate	Human	+		+	+	+	31	–
Bronchial wash	Human	+	A	+	+	+	34	–
Upper lung	Human	+	A	+	+	+	20	–
Lower lung	Human	+	A	+	+	+	26	–
Liver	Human	+	A	+	+	+	29	–
Spleen	Human	+	A	+	+	+	31	–
Pleural fluid	Human	+	B	+	+	+	–	36
Blood	Human	+		+	+	+	–	38
Spleen	Human	–		–	–	–	–	–
Liver	Human	–		–	–	–	–	–
Cerebrospinal fluid	Human	–		–	–	–	–	–
Blood	Human	–		–	–	–	–	–
Liver/spleen	Tamarin	+		+	+	+	28	–
Tissue	Tick¶	+	A	+	+	+	26	–
Tissue	Tick¶	+	A	+	+	+	33	–
Blood	Prairie dog	+	B	+	+	+	–	30
Blood	Prairie dog	+	B	+	+	+	–	27
Spleen	Prairie dog	+	B	+	+	+	–	21
Spleen	Prairie dog	+	B	+	+	+	–	31
Spleen	Prairie dog	–		–	–	–	–	–
Liver	Cat	–		–	–	–	–	–
Liver	Rat	–		–	–	–	–	–
Spleen	Rat	–		–	–	–	–	–
Spleen	Squirrel	–		–	–	–	–	–

**F. tularensis* infection identified by culture, direct fluorescent antibody testing, or serologic testing.
†Subspecies was determined by glycerol fermentation when an isolate was recovered.
‡+ = positive result, $17 \leq C_t \leq 38$; – = negative result, no fluorescence detected after 45 cycles of amplification.
§C_t, cycle threshold.
¶Tick species tested were *Haemaphysalis leporispalustris* and *Dermacentor andersoni.*

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Concurrent *Plasmodium vivax* Malaria and Dengue

To the Editor: The first report of a patient with concurrent malaria (*Plasmodium falciparum*) and dengue was recently published in this journal (*1*). Herein is presumably the first report of concurrent dengue and malaria due to *P. vivax*.

A 27-year-old woman experienced the onset of myalgia on December 11, 2003, 1 day before returning home to California from India after a 3-month sojourn in that country. The following day she had chills and a low-grade fever, and she visited an urgent care center. A presumptive diagnosis of influenza was made, and she was discharged with antipyretic therapy. A single malaria smear was subsequently reported to be negative for *Plasmodium*.

On December 15, she sought treatment at a hospital emergency department at 3:30 A.M. with an oral temperature of 39.5°C. Her leukocyte count was 4,300 × 10^9/L hemoglobin level 119 g/L, and platelet count 157,000 × 10^9/L. A diagnosis of probable viral syndrome was made, and she was discharged with antipyretic therapy. She returned to the urgent care center the following day with a temperature of 38.6°C, and a 10-day course of amoxicillin was prescribed on discharge.

On December 18, she sought treatment from an infectious disease specialist. She had an oral temperature of 39.3°C and was dehydrated, which led to her admission to the hospital. Results of the examination were otherwise unremarkable.

She reported that she had lived in the United States for the last 4 years, after moving there from India. During her recent trip to India, she had spent most of the time in Surat, followed by 3 days in Mumbai. She indicated that she had had malaria several times while living in India. She received no vaccinations before her trip and took no malaria prophylaxis; she believed she was likely immune and, in addition, she was concerned about taking medications while breastfeeding her 6-month-old child. The child received no prophylaxis or other medical preparation for the trip but remained well.

Her leukocyte count was 4,500 × 10^9/L with 50% polymorphonuclear leukocytes, 18% band forms, 3% myelocytes, and 1% metamyelocytes. Hemoglobin level was 11.1 g/L, and platelet count was now 98.0 × 10^9/L. *P. vivax* was seen on blood smear, and the patient was treated with chloroquine with rapid resolution of her fever, followed by administration of primaquine, during which course she avoided breastfeeding. In addition, enzyme immunoassays for dengue virus were performed on December 19 (immunoglobulin G [IgG]) 6.55; IgM 4.17) and subsequently repeated on December 31 (IgG 7.29; IgM 1.07), indicating an acute infection. Viral isolation was not attempted.

I agree with Charrel and colleagues (*1*) that, although only 2 cases have now been reported, concurrent dengue and malaria is probably not a rare event. This conclusion is supported by a recent report from Pakistan (*2*).

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Viruses from Nonhuman Primates

To the Editor: I read with interest the article by Jones-Engel et al. (*1*), which described the frequency of viruses infecting temple rhesus macaques. The investigation included the polyomavirus simian virus 40 (SV40), a pathogen recognized to have infected millions of humans who were vaccinated with polio vaccines produced in cultures of rhesus monkey kidney cells (*2*,*3*). The authors indicated that technologic advances have improved the specificity of detecting SV40 antibodies and used an enzyme immunoassay based on viruslike particles (VLPs) to perform the analysis (*1*). However, the specificity of the SV40 enzyme immunoassay is problematic because studies with serum samples from macaques have found that antibodies are cross-reactive with polyomaviruses JCV and BKV (*4*). In addition, in monkey sera SV40 VLPs correlated with BKV antibodies. Similar conflicting results have been found in human studies that used polyomavirus VLPs assays (*3*). These limitations are the result of polyomavirus VLPs assays using expression of the VP1 capsid protein (*4*), a highly homologous gene among JCV, BKV, and SV40 (*3*). In contrast, modern molecular biology assays are the preferred method for the analysis of SV40 infections (*2*,*3*). In addition, these sensitive and specific techniques can provide insights into the distribution of SV40 strains and variants (*2*,*3*). This is important because recent data suggest that the biological properties of SV40 strains vary in vivo (*5*).

Because current evidence shows that SV40 infections are identified in some humans and that the virus is associated with selected human malignancies (*2*,*3*), prospective longitudinal studies that use molecular

techniques are needed to examine the prevalence and ecology of SV40. The Institute of Medicine recognizes that the biologic evidence indicates that infections with this DNA virus could lead to cancer in humans and recommends targeted biologic research of SV40 in human populations (*2*).

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In response: Dr. Vilchez (*1*) raised questions regarding the specificity of the SV40 viruslike particle (VLP)–based ELISA used to detect SV40 infection in temple monkeys (*2*). Although it is true that SV40 infection can elicit low-level, cross-reactive antibodies that recognize polyomavirus BKV and to a lesser extent polyomavirus JCV VLPs and, conversely, BKV and JCV infection may elicit low-titer SV40 cross-reactive antibodies (*3*), these antibodies do not pose a problem for serologic diagnosis of infection in natural hosts of these polyomaviruses. Unless one were to hypothesize that BKV and JCV could infect macaques, SV40 VLP-reactive antibodies could not possibly be produced by anything other than an SV40 infection. In addition, SV40 seroreactivity in macaques is generally very strong but cross-reactive responses are weak (*3*).

Specificity of SV40 seroreactivity in macaques has recently been demonstrated by competitive inhibition assays (*4*). SV40 reactivity was blocked by incubation of sera with SV40 VLPs but not significantly reduced by incubation with BKV or JCV VLPs. Specificity of BKV and JCV VLP reactivity in human sera has also been demonstrated by preabsorption with VLPs (*5*) and competitive inhibition assays (*4*). Thus, BKV VLP seroreactivity can be completely inhibited by BKV VLPs but not by JCV VLPs and vice versa. When polyomavirus VLP ELISAs are used to diagnosis cross-species infection, such as SV40 infection in humans, competitive inhibition assays are necessary to verify specificity of the response. Engels et al. (*6*), using SV40 VLP serology and competitive inhibition assays, recently reported evidence for possible infection of zoo workers with SV40. We agree with Dr. Vilchez that molecular biology assays, such as PCR, play a valuable role in viral diagnosis. However, these assays also have limitations in terms of sensitivity and specificity and therefore are best combined with the full range of viral diagnostic techniques to confirm infection. Serologic testing is more suitable than other assays for estimating cumulative infection.

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Rickettsia parkeri in Uruguay

To the Editor: During 1990 in Uruguay, a rickettsiosis in the spotted fever group was presumptively diagnosed for 3 patients who had fever, an initial small maculopapulous lesion at the site of a tick bite on the scalp, and subsequent regional lymphadenopathy. Microimmunofluorescent serologic assay, with *Rickettsia conorii* as the sole antigen source, gave positive results for all patients, and these infections were presumptively identified as spotted fever caused by *R. conorii* (*1*). During 1993–1994, a total of 23 patients who had a history of tick bite, some of whom had exanthema and inoculation eschars, were identified from Canelones County, Uruguay. These patients had antibodies against *R. conorii*, according to microimmunofluorescence testing; however, *R. conorii* was again the sole antigen source used in the assay (*2*). Because 1 of the major limitations of serologic testing for diagnosis of rickettsioses is the cross-reactivity between different *Rickettsia* species, the association of *R. conorii* with the spotted fever group cases in Uruguay was considered inappropriate (*3*). In addition, *R. conorii* has never been found in the Western Hemisphere (*3*).

Amblyomma triste, a neotropical tick species with a variety of hosts, is the main tick species that feeds on humans in Uruguay and the primary candidate vector for tickborne rickettsioses in that country (*4*). A recent investigation demonstrated DNA of *R. parkeri* in *A. triste* ticks collected from humans and animals, indicating that this rickettsia could be the pathogenic agent of spotted fever group rickettsioses in Uruguay (*5*). In the United States, where *A. maculatum* ticks infected with *R. parkeri* have been reported since the 1930s, the role of this rickettsial agent as a human pathogen was confirmed only recently (*3*). Our study is the first to isolate *R. parkeri* from *A. triste* collected in Uruguay and confirms the presence of this emerging pathogen in South America.

During September 2004, 78 adult flat ticks (25 males, 53 females) identified as *A. triste* were collected from vegetation in the suburban area of Toledo Chico (34°44′53″S, 56°06′ 19″′W) in Canelones County, southern Uruguay. At the laboratory, the legs of live ticks were extirpated for DNA extraction, and the tick bodies were immediately frozen at –80°C. Each group of legs from 1 tick was subjected to DNA extraction by boiling at 100°C for 20 min as described (*6*). DNA extracted from each tick was tested by PCR by using primers CS-78 and CS-323 (Table), which targeted a 401-bp fragment of the citrate synthase gene (*gltA*) of possibly all *Rickettsia* species (*7*). For 2 ticks (1 male, 1 female) that had positive results with PCR testing, *Rickettsia* isolation in cell culture was attempted by using the shell vial technique with the following modifications: Vero cells inoculated with tick body homogenate were incubated at 28°C; the level of infection of cells was monitored by Gimenez staining of scraped cells from the inoculated monolayer; and a rickettsial isolate was considered established after 3 passages, each reaching >90% of infected cells (*7*).

Rickettsiae were successfully isolated and established in Vero cell culture from the female tick. This isolate, designated as At5URG, has been deposited as a reference strain in the Rickettsial Collection of Faculty of Veterinary Medicine in the University of São Paulo. DNA extracted from infected cells of the third passage was tested by a battery of PCRs that used all primer pairs listed in the Table and targeted fragments of 3 rickettsial genes: *gltA*, *ompB*, and *ompA*. PCR products of expected size were obtained in all reactions and subjected to DNA sequencing as described (*6*). Fragments of 1,084, 775, and 491 nt of the *gltA*, *ompB*, and *ompA* genes, respectively, were obtained and showed 100% identity to the corresponding sequences available in GenBank (accession nos. U59732, AF123717, and U43802, respectively) for the Maculatum strain of *R. parkeri* from United States. Although isolation of *Rickettsia* from the male tick was unsuccessful, DNA extracted from remnants of the male and female ticks was tested by PCR (*ompA*, Table) and yielded product that after

Table. Primer pairs used for amplification of rickettsial genes

Primer pairs	Genes and primers	Primer sequences (5′→3′)	Fragment size (nucleotides)	Reference
	gltA			
1	CS-78	GCAAGTATCGGTGAGGATGTAAT	401	*7*
	CS-323	GCTTCCTTAAAATTCAATAAATCAGGAT		*7*
2	CS-239	GCTCTTCTCATCCTATGGCTATTAT	834	*7*
	CS-1069	CAGGGTCTTCGTGCATTTCTT		*7*
	ompB			
3	120-M59	CCGCAGGGTTGGTAACTGC	862	*8*
	120–807	CCTTTTAGATTACCGCCTAA		*8*
	ompA			
4	Rr190.70p	ATGGCGAATATTTCTCCAAAA	530	*9*
	Rr190.602n	AGTGCAGCATTCGCTCCCCCT		*9*

sequencing (491 nt) showed 100% identity to the *R. parkeri* sequence from GenBank (U43802).

These procedures enabled the identification of *R. parkeri* in 2.56% of the *A. triste* ticks from Uruguay. Previous findings of *R. parkeri* DNA in *A. triste* ticks from Uruguay (*5*) are corroborated by our isolation of a Uruguayan strain of *R. parkeri* in cell culture. The only other country where *R. parkeri* has been previously reported is the United States, where it is associated with *A. maculatum* ticks and is the causative agent of an emerging rickettsiosis (*3*). As *A. maculatum* and *A. triste* are established in at least 12 other Latin American countries (*10*), the distribution of *R. parkeri* in the Americas is likely continental. Finally, our results corroborate recent reports (*3*,*5*) that suggest *R. parkeri* is the causative agent of previously reported cases of rickettsiosis in Uruguay.

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Influenza-related Death Rates for Pregnant Women

To the Editor: Articles about influenza in the January 2006 issue of Emerging Infectious Diseases discussed a pandemic possibly as profound in its effect as the 1918–19 pandemic, when attack rates were >20% worldwide and death rates were 1%–2%. Then, as when subsequent virus antigenic shifts have occurred, all age groups were affected. Governments are now preparing contingency plans against the effects of an expected further antigenic shift.

However, insufficient consideration may have been given to how, in the absence of effective prophylaxis against a novel strain of influenza virus, to avoid deaths on the scale seen in the fall and winter of 1918–19. In particular, the vulnerability of pregnant women and their offspring appears to have been forgotten. Bland reported on pregnant influenza patients in Philadelphia and elsewhere in the fall of 1918; of 337, 155 died (*1*). Harris obtained by questionnaire from obstetricians medical histories of 1,350 pregnant patients in Maryland and in 4 large US cities (*2*). Pneumonia developed in half (678) of these patients and 365 died. Death rates from pneumonia were >40% for every month of pregnancy; fetal loss

Correction: Vol. 12, No. 10

In Human Prion Disease and Relative Risk Associated with Chronic Wasting Disease by Samantha MaWhinney et al., an error occurred in the list of references. Missing from the list is reference no. 36: Belay ED, Maddox RA, Gambetti P, Schonberger LB. Monitoring the occurrence of emerging forms of Creutzfeldt-Jakob disease in the United States. Neurology. 2003;60:176-81.

The corrected list of references appears in the online article at http://www.cdc.gov/ncidod/EID/vol12no10/06-0019.htm

We regret any confusion this error may have caused.

was >40% in all months but the fifth (37%).

According to a contemporaneous report from England, the influenza death rate for pregnant women was 25.4% (*3*). These inquiries into pregnancy must have been biased toward severe cases, but the influenza pandemic in 1918–19 may nevertheless have decreased live births in England and Wales, which reached new lows in the first half of 1919 (*4*). A controlled American study during 1975–1979 has since confirmed that pregnant women are at risk for influenza even in interpandemic years (*5*).

After an interpandemic interval >35 years, any antigenic shift may again seriously affect young adults, including many pregnant women. Preparedness should therefore ensure the availability of timely and comprehensive management of influenza during pregnancy.

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OIE/FAO International Scientific Conference on Avian Influenza

Alejandro Schudel and Michel Lombard, editors

Karger, Basel, Switzerland, 2006
ISSBN: 3-8055-8031-2
Pages: 278; Price, US $236.50

Since 1997 the international community has witnessed a strain of highly pathogenic H5N1 avian influenza virus emerge and spread at an unprecedented rate. It has had devastating consequences for domestic poultry, wild avian species, and humans on 3 continents; 240 human cases have occurred with 141 deaths. From 1999 to 2003, poultry outbreak control measures in the European Union alone resulted in the depopulation of 50 million birds at a substantial cost to the global economy. Because of the ongoing human and animal infections, the public health and veterinary communities have recognized the urgent need for an ongoing collaborative and participatory approach to prevention and control of highly pathogenic avian influenza (HPAI).

This monograph contains the proceedings of the International Scientific Conference on Avian Influenza held in Paris, France, in April 2005. The conference was sponsored by the World Organization for Animal Health (OIE) and the Food and Agriculture Organization (FAO), in collaboration with the World Health Organization (WHO). To address the emerging animal health crisis and mitigate risks to human health, at the outset of the HPAI H5N1 outbreaks in Southeast Asia in early 2004, these organizations held joint meetings in Rome (February 2004), followed by 2 regional meetings in Bangkok (February 2004) and Ho Chi Minh City (February 2005) to issue guidelines and recommendations for prevention and control. Because it appears that this HPAI H5N1 epizootic will persist for some time, the Paris 2005 meeting was held to achieve consensus on the most current strategies for long-term prevention and control, including poultry vaccination when appropriate.

Bernard Vallat, the director general of the OIE, opened the meeting and urged scientists and regulators to consider strengthening farm biosecurity measures; to assess the role of ducks as a reservoir for avian influenza; to evaluate animal vaccination strategies; and to promote strengthening of veterinary services to enable better detection, surveillance, and response as an "international public good." This meeting marked the official launching of the OIE/FAO Network (OFFLU), a network of avian influenza reference laboratories created to promote research on avian influenza, provide technical assistance to developing countries on diagnosis and management, and serve as a mechanism to interface with the WHO Influenza Network to obtain virus isolates from animals that can be used to produce vaccines to prevent a human pandemic.

Opening remarks were made by Ilaria Capua, director of OIE and the National Reference Laboratory for Newcastle Disease and Avian Influenza in Padua, Italy. Dr. Capua called on the veterinary scientific community to take the following actions to limit the spread of the outbreaks: 1) expand understanding of the role of waterfowl and other nongallinaceous birds in the ecoepidemiology of HPAI, 2) further define the role of poultry vaccination in reducing the spread of infection and promoting animal welfare, 3) educate workers about prevention of exposure to avian influenza, and 4) conduct studies to address food safety concerns.

More than 300 internationally renowned scientists with expertise in avian influenza attended the meeting, which featured sessions on ecology and epidemiology, pathogenesis, human health implications, diagnostics, control strategies including vaccination, and improvement of management tools. Highlights of the scientific recommendations generated include an emphasis on global sharing of viral isolates, research on epidemiology of wild birds, research on mechanisms of transfer between wild and domestic avian species, and research on pathogenesis in other farmed birds to clarify their role as intermediate hosts. The scientists concluded that the following elements were critical for achieving long-term control of HPAI infections in animals and humans: monitoring viruses for antigenic changes in virulence, performing surveillance of H9N2 viruses with the potential to infect mammals, and conducting epidemiologic studies at the human-animal interface by the OIE/FAO and the WHO networks of reference laboratories

This monograph contains the full text of the introductory speeches and manuscripts upon which the invited talks and abstracts of the poster sessions were based. It is an excellent reference for anyone interested in understanding the challenges the public health and veterinary community are facing due to the rapid emergence and complex ecoepidemiology of a viral pathogen that represents a major threat to public health and animal well-being.

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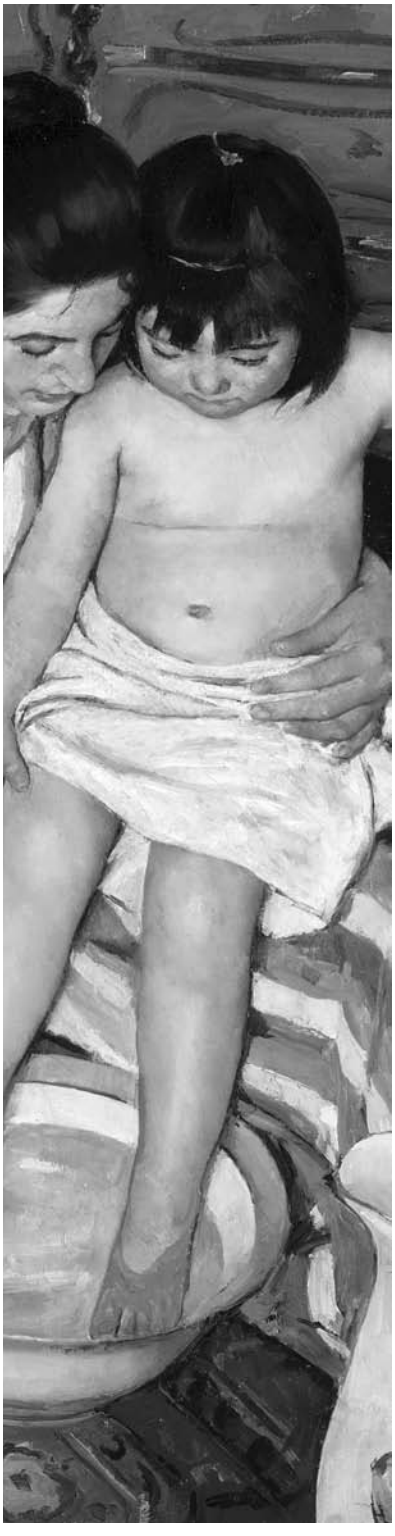

Mary Cassatt (1844–1926)
The Child's Bath (1893)
Oil on canvas (100.3 cm × 66.1 cm)
Robert A. Waller Fund, 1910.2
Reproduction, The Art Institute of Chicago, Chicago, Illinois, USA

Women Caring for Children in "the Floating World"

Polyxeni Potter*

"A figure of a woman, is lining up a billiard shot, while the figure of a man…stands dreaming in a doorway," writes artist-printmaker Peter Milton (b. 1930). He is describing the photograph by Gertrude Kasebier in 1908, which inspired his print Mary's Turn. "…it was the drama of the purposeful woman and the pensive man which established the direction Mary's Turn was to take" (*1*). The pensive man was Edgar Degas; the purposeful woman none other than Mary Cassatt, grand dame of impressionism.

Born to an affluent Pennsylvania family, Cassatt enjoyed a privileged childhood and cultural opportunities at home and abroad. Independent and strong willed, she prevailed upon her reluctant parents to let her enroll, at age 16, in the Pennsylvania Academy of Fine Arts in Philadelphia. Against convention, she soon resolved to pursue an artistic career and set off to Paris to study the Old Masters. When the Franco-Prussian War broke out in 1870, she returned briefly to the United States but left again to travel in Italy, Spain, and Belgium and to finally settle in Paris, where she lived the rest of her life.

In the late 1800s, Paris was the center of the art world. Its architecture and transportation system set the standards for 20th-century urban living. Émile Zola described the travails of common people, Claude Debussy found new musical expressions, and French politics was undergoing a democratic revolution. Cassatt set up her studio and studied with academic painter Jean-Léon Gérôme. Her early work was influenced by the realism of Gustave Courbet and Eduard Manet (*2*). She sent her work to the Salon, an annual showcase that judged art on its adherence to agreed upon subjects and strict rules. In 1868, her painting The Mandolin Player was accepted.

The rigid rules of the Salon and passion for creative freedom drove artists to independent exhibits. "I only began to live," Cassatt asserted, "…at the moment Degas persuaded me to… exhibit with his friends in the group of Impressionists. I accepted with joy….I hated conventional art" (*3*). The only American painter to do so, she exhibited often with the impressionists, and under their influence she revised her approach to composition, color, and light, showing admiration for the group, especially Degas.

"It's true. There is someone who feels as I do," Degas once exclaimed in front of one of Cassatt's paintings (*4*). On her part, Cassatt maintained that the first sight of Degas' pastels "was the turning point in my artistic life" (*4*). The two became lifelong friends, supported and influenced each other, and painted portraits of each other. "Oh, my dear, he is dreadful!" Cassatt once confided to her friend, art patron Louisine Havemeyer. "He dissolves all your will power" (*1*). Degas' cantankerous behavior eventually ended the friendship, even though in their old age, both produced great work until both went blind and became unable to paint.

"It is essential to do the same subject over and over again, ten times, a hundred times," advised Degas (*5*). Such intensive involvement with a subject also marked Cassatt's work. Her models were family and friends sitting in the loge at the opera, taking tea, reading, knitting. Over 6 years, she painted more than 20 works exploring the lives of women and their close relationships with children.

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"You who want to make color prints wouldn't dream of anything more beautiful….You must see the Japanese," wrote Cassatt to fellow impressionist Berthe Morisot, after visiting an exhibition at the École des Beaux-Arts (*6*). Woodblock prints by such masters as Kitagawa Utamaro and Katsushika Hokusai provided unprecedented views of traditional *ukiyo-e*, scenes of the floating world (everyday life). Their directness, linear elegance, compositional strength, and tonal richness so impressed and inspired Cassatt that she turned to printmaking. She invented her own techniques and adopted Japanese aesthetics to convey the private mood and intimacy of her domestic scenes.

"I suppose it is…Palmer's French blood which gives her organizing powers and determination that women should be someone and not something," reflected Cassatt about the exceptional qualities of Bertha Honoré Palmer, business woman and philanthropist (*7*), who invited her to paint the south tympanum in the Women's Building at the World's Columbian Exposition in Chicago. The theme, "Modern Woman," was a tribute to women's education, "Young Women Plucking the Fruits of Knowledge and Science." Cassatt so feared the judgment of Degas lest he "demolish me so completely that I could never pick myself up in time to finish for the exposition" that she did not show him the work in progress. On his own part, Degas said of Modern Woman, "I will not admit a woman can draw like that!" (*1*).

Americans were the first patrons of the impressionists, amassing substantial private and museum collections. Cassatt was a frequent advisor to collectors of both Old Masters and the avant-garde. When Louisine Havemeyer sought advice about a New York exhibition in 1915 showing paintings by Cassatt and Degas, as well as by Holbein, Rembrandt, and Vermeer, she advised, "…put a Vermeer of Delft near the Degas and let the public look first at the one and then at the other. It may give them something to think about" (*1*).

"I doubt if you know the effort it is to paint! The concentration it requires, to compose your picture, the difficulty of posing the models, of choosing the color scheme, of expressing the sentiment and telling your story" (*8*). Cassatt was highly skilled. She preferred to work with unposed models placed in asymmetric settings, seen from unusual vantage points. She flattened forms and perspective, contrasted colors and decorative patterns, and used background to establish spatial relationships and shift the focus of perception.

The Child's Bath, on this month's cover, is characteristic of Cassatt's mature work and elaborates on her preferred theme: women caring for children. Preference for the theme reflects her own affection for children and knowledge of 19th-century child-rearing practices. Several cholera epidemics in the mid-1880s prompted official promotion of regular bathing as prevention against disease. And after 1870, French mothers were encouraged to take care of their own children, instead of employing caretakers, and to use modern hygiene practices (*9*).

Cassatt captures a private moment between a woman and a child. The two are absorbed in a domestic ritual, looking down, heads touching, arms interlocked. Aligned along strong diagonals, chubby legs boldly cross ample striped dress, in sharp contrast with circular shapes: heads, washbasin, pitcher. She gently rubs the small foot with one hand, the other holding the child securely in her lap. Lips are parted imperceptibly. Perhaps she is explaining the reflections inside the washbasin. The tender moment, is punctuated by the surroundings: a painted chest-of-drawers, placing the activity on the floor, from the child's perspective, while we have the oblique view from the top. Flowered wallpaper and portions of decorative carpet define the cropped edges of the composition.

"Even more important than the discovery of Columbus which we are gathered here to celebrate," said Bertha Honoré Palmer in her speech on the opening day of the World's Columbian Exposition in 1893, "is the fact that the general government has just discovered women." Though times have changed, Mrs. Palmer's words still ring true in much of the world. Caregiving and safeguard of the physical and emotional health of children go beyond the hygienic benefits of the bath and are tightly connected with the physical and emotional health of the caregiver. To remedy long neglect of the caregiver and protect against emerging health threats, it is time, as Cassatt put it, for women to pluck the fruits of knowledge and science.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Look in the December issue for the following topics:

Ecologic Niche Modeling and Spatial Patterns of Disease Transmission

Review of Bats and Severe Acute Respiratory Syndrome

Cattle Grazing and Risk for Lyme Disease

Isolation of Lagos Bat Virus from Water Mongoose

Leishmania tropica in 2 Adjacent Foci, Northern Israel

Risk Factors for Human Avian Influenza (A/H5N1) Infection, Vietnam, 2004

Human African Trypanosomiasis Transmission, Kinshasa, Democratic Republic of Congo

Fatal Human Infection with Rabies-related Duvenhage Virus, South Africa

Cross-Species Transmission of Human and Macaque *Plasmodium* sp. to Wild-Born Orangutans, Indonesia

Zoonotic Focus of Plague, Algeria

Borrelia garinii in Seabird Ticks (*Ixodes uriae*) from North American Atlantic Coast

Complete list of articles in the December issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

February 23–25, 2007
IMED 2007: International Meeting on Emerging Diseases and Surveillance
Vienna, Austria
Contact: info@isid.org or 617-277-0551 voice; 617-278-9113 fax
http://imed.isid.org/

March 7–9, 2007
6th International Symposium on Antimicrobial Agents and Resistance (ISAAR 2007)
Raffles City Convention Centre Singapore
http://www.isaar.org

March 20–23, 2007
ISOPOL XVI: 16th International Symposium on Problems of Listeriosis
Marriott Riverfront Hotel
Savannah, GA, USA
Abstract submission deadline: November 1, 2006
Contact: 240-485-2776
http://www.aphl.org/conferences/ISOPOL.cfm

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
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The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

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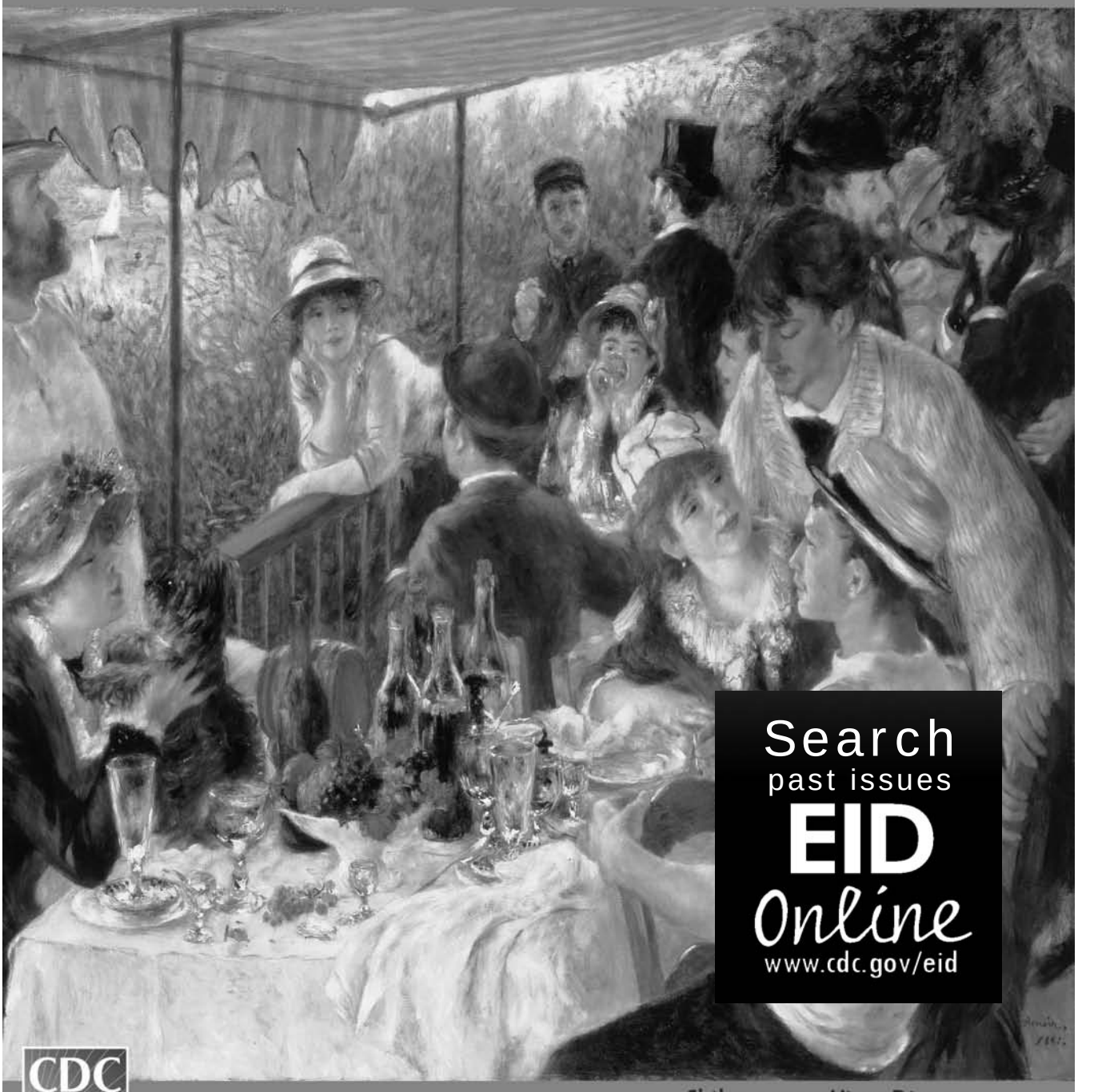

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Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje. org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by “et al.” Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word’s table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only 2. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.